STUDIES ON THE FLOCCULATION REACTIONS OF SERUM PROTEINS

BY

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INTRODUCTION

The alterations of the serum proteins which are important from the clinical point of view may be recorded by precise, but often laborious and timeconsuming methods. In the clinical laboratory, however, other simpler and more rapid techniques are commonly used. This applies especially to a group of screening tests that are called flocculation or lability reactions. They are first and foremost used as tools in the differential diagnosis of jaundice and to establish the prognosis of liver diseases, and they are commonly regarded as sensitive indicators of liver parenchyma damage. It is not claimed, however, that they are specific for liver diseases. The use of these methods is empirical, and knowledge of their biochemical and physico-chemical background is incomplete in several aspects.

In the widest sense of the word "lability", every reaction in which serum is introduced into an environment where a precipitation of the proteins occurs, may be called a lability reaction. The numerous tests belonging to this category have been exhaustively reviewed by Heepe (1953) and also by Popper and Schaffner (1957). In liver parenchyma diseases the serum proteins show an "increased lability". The expression "decreased stability" would perhaps be more in accordance with the nomenclature of general macromolecular chemistry, in which the conception of the stability of colloids is widely discussed (see for instance Overbeek 1952 a). As a matter of fact, several of the reactions discussed here can also record a subnormal precipitation, i.e. an increased stability.

It is a common experience that the flocculation reactions all give more or less parallel results although the procedures are extremely variable. It has therefore been suggested that they reflect principally the same changes of the serum constituents. Although the influence of non-protein substances have been discussed, the general view at present is, that the main factors are to be found within the serum proteins. The intention of this investigation has been to elucidate the mechanism of the flocculation reactions; in the author's opinion the most important aspect of this subject is the interaction of the serum proteins with each other. In part I of this thesis some indirect evidence of this interaction is presented, where some proteins act by being precipitated, others by inhibiting the process of precipitation. In part II the protein-protein interaction will be studied in a more direct way by following the change of solubility which takes place in isolated serum proteins when mixed with other proteins.

Part I

THE FLOCCULATION REACTIONS AND THE ELECTROPHORETIC PATTERN OF THE SERUM PROTEINS

Chapter I. Serum protein changes in liver diseases Review of the literature

A. *Early investigations*

The early literature on this topic has been thoroughly reviewed by Gutman (1948). Apart from some observations on the lowered colloid osmotic pressure of serum (for references see von Farkas 1938) it was not until 1929 that Abrami and Wallich, and Salvesen, demonstrated that the albumin/globulin quotient as determined by fractionation with neutral salts, was lowered in liver cirrhosis. D'Antona and Pellegrini in 1934, and Gros in 1937 first showed by further fractionation with neutral salts that the increase of globulins primarily concerned that part of the globulin which was precipitated at low concentrations of salts.

B. *Alterations in the electrophoretic pattern of the serum proteins in liver disease*

The introduction of *moving boundary electrophoresis* by Tiselius and its application in the clinical laboratory opened new ways for a detailed analysis of changes of serum proteins. The first electrophoretic investigation on serum proteins in liver disease was performed by Longsworth and Shedlovsky (1939). It was a case of obstructive jaundice which showed a great increase of β-globulin. After extraction of the serum with ether most of the β-peak disappeared. In table 1 some of the principal works on moving boundary and paper electrophoresis of serum proteins in liver diseases are presented. The results must always be compared with the normal values from the same laboratory since small variations in technique yield different figures. The author's own material is presented in the last column. To sum up, the following changes are regularly found (cf. also Luetscher 1941, Gray and Barron 1943, Kabat, Hanger and co-workers 1943, Malmros and Blix 1946, Thorn, Armstrong and Davenport 1946, Cohen and Thompson 1947, Whitman, Rossmiller and Lewis 1950, Wunderly, Karrer and co-workers 1951, Charbonnier,

Baesens and Caroli 1953, Poli, Caspani and co-workers 1953, Marner 1955): in liver cirrhosis a decrease in the content of albumin and a simultaneous increase of the content of γ-globulin is invariably observed. Often, but not always, an increase of β-globulin is also observed. As regards α_2-globulin opinions do not quite agree. Some investigators report an occasional increase, others have found an α_2-globulin within the normal range. Wunderly, Karrer *et al.* (1951) remark in their detailed report on clinical states with increased α-globulin: "Fait intéressant, nous ne trouvons jamais d'hyperglobulinémie α dans la cirrhose du foie, à moins qu'elle soit accompagnée de quelques complications. Une élévation du taux relatif des globulines α au-dessus de 10 % est un indice certain d'une complication indépendante de l'affection hépatique." In liver cirrhosis there has often (in about half the cases according to Flynn 1954) been observed a considerable increase of the fast moving component of γ-globulin, also called γ_1- or H-globulin (Viollier 1951, Franklin, Bean *et al.* 1951 b). In some cases this increase causes difficulties in differentiating between β-globulin and γ-globulin in the electrophoretic pattern. An increase of γ_1-globulin, however, also occurs in other clinical conditions (see for instance Barbagallo-Sangiorgi 1953). The remarkable augmentation of the β-fraction in the biliary type of liver cirrhosis has especially been pointed out by Kunkel and Ahrens (1949) and Ricketts and Sterling (1949b).

As regards the cases of acute hepatitis, the protein pattern seems to be principally the same as in liver cirrhosis, even if the changes are not equally pronounced, i.e. an increase of γ-globulin and in most cases also an increase of β-globulin, a decrease of albumin and an inconstant increase of α-globulin (cf. also Martin 1946, Viollier 1951). The largest material is presented by Hartmann (1950). His cases show on an average a content of α-globulin within the normal range and a slight increase of β-globulin, but on the other side he found an increase of α- and β-globulins initially and also during the remission of the disease, as did also Rafsky, Weingarten *et al.* (1950). Zöllner and Eymer (1950) observed in infective hepatitis an initial increase of β-globulin and γ-globulin and a decrease of α-globulin. In a recent paper Wajchenberg, Hoxter and co-workers (1956) in cases of fatal diffuse hepatic necrosis found a highly significant reduction in albumin and β-globulin, a significant reduction in α_2-globulin and a significant increase of γ-globulin compared with the normal control group.

The pattern in obstructive jaundice is best presented by Popper, Bean *et al.* (1951) with a decrease of albumin, an increase of α- and β-globulin and a slight increase of γ-globulin.

The development of *paper electrophoresis* as a simple and inexpensive method has to an extraordinary extent enriched the facilities for studying serum proteins. A great many works have been published on the topic of liver diagnostic methods and only the principal points will be related

here. Generally speaking, the results are in accordance with those obtained by the free electrophoresis, i.e. in cases of liver parenchyma lesions a decrease of albumin and an increase of γ-globulin is always seen. The more severe the liver cell damage, the more pronounced will these changes be (Satoskar, Lewis *et al.* 1954, Broicher and Odenthal 1954, Ceschka, Rissel and Wewalka 1956). With the method of paper electrophoresis there is also better agreement with the intermediate globulin fractions. The β-globulin content is often quite normal. This is at variance with the results obtained with the Tiselius electrophoresis, as has also been emphasised by others (Olhagen 1955). The α_2-globulin is normal or even low. Already in 1952, Brante laid stress upon the fact that the α_2-globulin in hepatitis and the α_2- and β-globulins in liver cirrhosis were unaltered. In the last-mentioned disease he even observed subnormal values of these intermediate fractions. Subnormal values of α_1- and α_2-globulin were also recorded by Satoskar, Lewis *et al.* (1954) in severe cases of viral hepatitis and portal cirrhosis. Flynn (1954) observed a decrease of α_1-, α_2- and β-globulins in acute hepatic necrosis. Ceschka (1956) writes about liver cirrhosis: "Eine Vermehrung der α-Globuline ist als abnorm zu betrachten. Sie lässt Verdacht auf einen gleichzeitig bestehenden entzündlichen Prozess aufkommen." (cf. also Sonnet 1956). This author also introduced an index, the ratio α_2/γ as a measure of the degree of liver damage. This ratio amounted to 0.45 in normal cases, in cirrhotic livers it dropped down to 0.19. A recent contribution from von Studnitz (1956) also emphasises the subnormal contents of α_2-globulin in cases of cirrhosis and hepatitis. Krook (1956) often found decreased α_1-, α_2- and β-globulins in his large material of liver cirrhoses. In contrast to these findings on the α_2-globulin are the observations by Knedel (1951) and Flynn (1954) on acute hepatitis and Küchmeister and Voigt (1954) on chronic hepatitis and incipient cirrhosis.

In obstructive jaundice, on the other hand, there is very often found an increased α_2-globulin content (Brante 1952, Owen and Robertson 1956). The increased β-globulin emphasised by Raynaud, Miniconi *et al.* (1954) was never found by Owen, not even in "cholangiolithic cirrhosis".

Thus a typical, though not specific, electrophoretic pattern has been described in liver parenchyma disease, and the evidence obtained by free electrophoresis and paper electrophoresis is concordant, with the exception of β-globulin. The higher content of β-globulin (and perhaps also sometimes of α_2-globulin) obtained with the Tiselius method may be explained by the changes in the lipoproteins that appear in liver disease and that are detected by the interferometric recording in this method (cf. also Mackay, Volwiler *et al.* 1954). The absence of increase of the α_2-globulins in infective hepatitis, as opposed to the increase found in most other acute diseases, for example cholecystitis, seems to be a rather characteristic feature of liver parenchyma disease, and this tendency culminates in the very low values found in acute hepatic necrosis.

At last it may be mentioned that Miller and Bale (1954) have demonstrated in animal experiments, firstly the synthesis in the liver not only of albumin but also of all globulins except γ-globulin, and secondly, a more rapid turnover of α-globulins than of any other plasma protein fraction.

TABLE. 1. *The electrophoretic pattern of the serum proteins in liver diseases.*

(1) *Moving boundary electrophoresis*

	No. cases	Relative values (%)					Absolute values (g/100 ml)				
		Albumin	Globulin				Albumin	Globulin			
			α_1	α_2	β	γ		α_1	α_2	β	γ
Ricketts & Sterling 1949											
normal	10	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93
virus hepatitis	10	43.9	6.7	11.7	17.6	20.2	2.78	0.41	0.77	1.14	1.27
biliary cirrhosis	10	40.6	6.2	11.6	22.0	19.6	2.89	0.44	0.81	1.60	1.44
Verschure & Janssen 1950											
normal function of the liver		60.0	7.4		11.8	20.8					
light disturbance		49.7	9.4		14.2	26.6					
moderate disturbance		43.5	12.6		14.1	29.8[a]					
severe disturbance		40.3	7.1		14.6	38.0					
terminal disturbance		30.0	4.6		10.3	55.0					
Hartman 1950											
normal	130	63.6	11.8		13.1	11.5					
hepatitis	131	52.7	10.3		15.0	22.0					
cirrhosis	65	38.0	10.5		15.0	36.5					
Franklin, Bean *et al.* 1951 a											
normal	10	60.1	5.0	9.3	11.5	14.1	4.40	0.37	0.68	0.84	1.03
infective hepatitis	6	45.1	5.2	10.8	16.6	22.3	3.47	0.40	0.83	1.28	1.72
cirrhosis	23	35.0	5.7	11.3	17.3	30.7	2.47	0.40	0.80	1.22	2.17
obstructive jaundice	3	43.5	5.7	16.4	16.8	17.6	3.13	0.41	1.18	1.21	1.27
Armas-Cruz, Lobo-Parga & Madrid 1952											
normal		57.6	7.4		14.3	20.7					
hepatitis		45.0	5.5		18.9	30.0					
cirrhosis		30.5	5.8		14.0	49.4					

[a] Some cases of metastatic carcinoma with high α_2-globulin.

TABLE 1 (*continued*).

(2) *Paper electrophoresis.*

	No. cases	Relative values (%)					Absolute values (g/100 ml)				
		Albumin	Globulin				Albumin	Globulin			
			α_1	α_2	β	γ		α_1	α_2	β	γ
G. Brante 1952											
normal	39	64.6	7.6		11.1	16.8	4.78	0.56		0.82	1.24
acute hepatitis	18	50.4	8.5		14.8	26.4	3.69	0.62		1.08	1.93
liver cirrhosis	9	40.8	6.3		10.0	42.8	2.90	0.45		0.71	3.04
cholelithiasis without inflammation	10	59.0	9.3		12.7	19.0	4.38	0.69		0.94	1.41
acute cholecystitis	14	57.0	12.6		13.9	23.2	4.03	0.89	0.89	0.98	1.64
Satoskar, Lewis & Gaitonde 1954 (plasma)						($\gamma + \varphi$)					($\gamma + \varphi$)
normal	10	57.5	4.2	6.4	10.2	21.7	4.50	0.33	0.50	0.80	1.70
virus hepatitis, acc. to icterus											
I	10	46.0	5.2	6.7	10.9	31.3	3.38	0.38	0.49	0.80	2.30
II	13	46.5	4.2	5.8	11.0	32.4	3.50	0.32	0.44	0.83	2.44
III	12	49.5	2.3	3.2	13.0	32.1	3.70	0.17	0.24	0.97	2.40
portal cirrhosis with ascites	27	32.7	3.1	5.9	11.5	46.7	2.10	0.20	0.38	0.74	3.00
Ceschka, Rössel *et al.* 1956											
normal	30	57.9	3.2	8.5	11.4	19.0	4.17	0.23	0.61	0.82	1.37 [b]
liver cirrhosis	100	38.5	3.1	7.2	11.1	38.5	2.67	0.22	0.49	0.83	2.64
Owen & Robertson 1956											
normal	34	65	5	6	9	15	4.6	0.4	0.4	0.6	1.1
infective hepatitis	21	53	5	7	13	22	3.8	0.4	0.5	0.9	1.6
diffuse hepatic fibrosis	12	31	6	6	9	47	2.0	0.4	0.4	0.6	3.0
acute hepatic necrosis	2	49	3	3	6	41	3.3	0.2	0.2	0.4	2.7
cholecystitis	9	50	6	10	12	23	3.4	0.4	0.7	0.8	1.5
stone in common bile duct	22	46	6	10	12	25	2.9	0.4	0.6	0.8	1.6
The author's values (method 1 B)											
normal	50	51.9	5.4	8.5	14.1	20.2	3.7	0.4	0.6	1.0	1.4
obstructive jaundice	28	40.4	8.4	13.2	14.6	23.4	2.9	0.6	0.9	1.1	1.7
infective hepatitis	28	41.6	5.6	8.1	13.2	30.1	2.9	0.4	0.6	1.0	2.1
liver cirrhosis	23	37.3	4.8	8.6	12.6	36.7	2.8	0.3	0.6	0.9	2.8

[b] Calculated on the assumption of a total protein content of 7.2 g/100 ml.

C. *Further aspects: Alterations in the lipoproteins and the glycoproteins of serum in liver disease. The occurrence of abnormal proteins.*

The lipoproteins

Deuel (1955) gives a summary of the serum lipids and their alterations in liver disease. Comprehensive data from the literature dealing with the lipoproteins is also provided by Nikkilä (1953), Marner (1955), and Eder, Russ *et al.* (1955). The value of the results obtained by means of analyses and fractionation of the total lipids has been somewhat limited. This must also be said of the earlier observations on the changes in the quotient of free cholesterol/esterified cholesterol, the differential diagnostic value of which in diseases of the liver has recently been queried by Albrink, Mann, and Peters (1950). In cases of advanced obstructive jaundice and biliary cirrhosis a high lipid value is constantly observed, with the phospholipids predominating (Reubi 1948a, Kunkel and Ahrens 1949). The last-named authors emphasise the fact that it is this predominance of the phospholipids which causes the serum to appear translucent, despite its high lipid content (Ahrens and Kunkel 1949). Cases in which the major injury is parenchymal, show a normal or subnormal cholesterol value. When the liver has been seriously damaged (Kunkel, Ahrens and Eisenmenger 1948), an extremely low total lipid value has been observed in the serum. In acute yellow atrophy of the liver the phospholipid values are also exceptionally low (Reubi 1948a).

The discovery and development of technical refinements in the analysis and preparation of serum proteins, has made it possible to undertake a more intensive investigation of the lipoprotein changes, associated with diseases of the liver. Kunkel and Slater (1952) separated the serum proteins on starch as a stabilising medium and recorded a most extraordinary finding; viz. that in some cases of biliary obstruction, α_1-lipoprotein is either completely absent, or, when present, is found in very low concentration. Nikkilä (1953) made similar observations when separating serum proteins on filter paper, but also found the same change in cases of liver parenchyma damage. Moreover, both these authors observed yet another qualitative alteration in β_1-lipoprotein, namely a comparative increase of the phospholipids. The observation that, in icterus, either caused by injury to the cells of the liver, or resulting from uncomplicated biliary stasis, the α_1-lipoprotein disappears, has been confirmed from several sources (Nys 1954, Wunderly and Piller 1954, Raynaud and d'Eshougues 1954, Benhamou and co-workers 1955, Klein and Franken 1955, Casassus and Cottet 1956) with the examination carried out by means of paper electrophoresis with lipoprotein staining of the paper. Several authors have reported that the α_1-lipoprotein reappears when the icterus has dispersed (Weicker 1955, Eder and co-workers 1955). Marner

(1955), who has separated serum lipoprotein by means of moving boundary electrophoresis, also stresses the fact that there is a correlation between the disappearance of the α_1-lipoprotein and the extent of the jaundice which means that this laboratory finding is not applicable to the differential diagnosis of icterus. Divergent findings have, however, been reported by Kushner, Dubin *et al.* (1956) and Nys and Vandenbroucke (1956), who have not been able to observe any close connection between bilirubinemia and the variations of the α_1-lipoprotein. Low α_1-lipoprotein content has also been reported in cases of cirrhosis of the liver by Berg, Scheiffart and Marwan in 1957. In this context the increase of β-globulin, which has been observed in free electrophoresis of serum in diseases of the liver, should be brought to mind. The fact that this increase may be ascribed most correctly to the lipid component has already been mentioned above (cf. Kunkel and Ahrens 1949).

Other observations, including some dating from earlier researches, also indicate that a disturbance of the interaction of the lipids and the proteins takes place in diseases of the liver. Serum lipids can be extracted by ether to a large extent, both in disorders of the biliary ducts and in those of the liver parenchyma. (Tayeau 1943, Reubi 1948b).

Somewhat divergent results have been obtained by Eder, Russ *et al.* (1955). There is, however, a crucial difference between their methods and those in general use. They have divided plasma from acute hepatitis, biliary cirrhosis and obstructive jaundice into three parts by means of fractionation with ethanol at low temperature (Cohn's method no. 10). In the fraction (IV + V + VI) which normally contains α_1-lipoproteins, lipoproteins of a kind were, admittedly, present, but were atypical inasmuch as the cholesterol component was non-esterified; they also moved in electrophoresis as the β-lipoproteins do. On the other hand, the β-lipoproteins in fraction (I + III) moved in electrophoresis in the typical manner, but their quotient of cholesterol/phospholipids was reduced, and there was also a decrease in the amount of esterified cholesterol.

The glycoproteins

Greenspan, Tepper *et al.* (1952) defined the quantity of mucoprotein (seromucoid, according to older terminology, or Winzler's orosomucoid) in serum under various conditions. They found an increase in value in cases of liver metastasis, a normal or increased value in biliary obstruction or inflammation of the biliary ducts, but on the other hand a subnormal value in the majority of cases of hepatitis or cirrhosis of the liver. These findings have lately been confirmed by Bang (1956), Björnesjö (1956) and others. This mucoprotein is said to move, for the most part, with α_1-globulin, in electrophoresis at alkaline pH.

Jayle and Boussier (1955) point out that the content in serum of haptoglobin, a glycoprotein moving as α_2-globulin, is low or normal in

uncomplicated liver parenchyma disease. The relative percentage of protein-bound carbohydrate in albumin, α_1- and α_2-globulin is subnormal in hepatitis and cirrhosis (Sonnet 1956). With regard to the *amount* of carbohydrate present in the various electrophoretic serum protein fractions in diseases of the liver parenchyma, the available information is at present scanty and sometimes contradictory. A certain increase in the albumin and α_1-globulin content of carbohydrate has been reported by Goa (1955), Puls and Fiedler (1956), Schaffner, Scherbel *et al.* (1956) and, earlier, in the mucoprotein content by Greenspan, Lehman *et al.* (1951). For γ-globulin, the reports do not tally with each other, but an increase in these elements has been observed by Laurell, Laurell and Waldenström (1956) and by Puls and Fiedler (1956).

The occurrence of abnormal proteins in liver diseases

In chapter II evidence is collected from the literature of the appearance in liver parenchyma disease of albumins and γ-globulins of an anomalous type with reference to their behaviour in flocculation reactions. In this section a short account is given on some investigations where the protein anomaly has been studied by other methods.

Serum proteins with an abnormally high molecular weight have been observed in cirrhosis of the liver by Martin (1949), Jahnke and Scholtan (1953) and Eriksen (1956). Cryoglobulins have been discovered in cirrhosis of the liver and chronic hepatitis by Wertheimer and Stein (1944), Barr, Reader *et al.* (1950), Griffiths and Gilchrist (1953) and a high-molecular cryoglobulin by Braunsteiner, Falkner *et al.* (1954). Such discoveries are, however, infrequent. Charlwood (1954) stresses the fact that albumin, isolated by electrophoresis from sera obtained from hepatitis and cirrhosis has a molecular weight completely in accordance with that of normal albumin.

Kunkel and Hoagland (1947) and Kunkel (1951) pointed out that albumin and γ-globulin isolated from liver diseases show no divergence from normal protein fractions under examination conducted according to serological methods.

Summary

Since diagnosis of liver disease is the most important field for the use of flocculation reactions, a survey of the literature on the protein changes in those diseases has been presented. Electrophoretic investigations have shown that in cases of damage of the liver parenchyma the sera are characterised by decreased albumin, normal or decreased concentrations of α_1- and α_2-globulins and increased concentrations of γ-globulin. In the differential diagnosis between infectious hepatitis and obstructive jaundice the changes in α_2-globulin have been particularly emphasised. The amounts of β-globulin

are increased in all kinds of liver diseases, if determined by moving boundary electrophoresis; but within the normal range, or, in parenchymatous damage, may even show a decrease if determined by paper electrophoresis. The observations on β-globulin made with moving boundary electrophoresis are confirmed by the analysis with other techniques on the lipoprotein in this fraction. The observed decrease of the α_1- and α_2-globulins may perhaps be correlated with the decreased mucoprotein level in damage of the liver parenchyma.

Chapter II. The participation of serum proteins in flocculation reactions; their promotion and prevention of precipitation

A. *Review of the literature*

1. The influence exerted on flocculation reactions by the electrophoretic fractions of the serum proteins

The effect of γ-globulin

In 1943 Kabat, Hanger *et al.* investigated γ-globulins isolated by electrophoresis from sera of normal patients and patients with hepatitis for their precipitating ability with the cephalin-cholesterol reagent. They found no difference between the two kinds of γ-globulins. Maclagan and Bunn (1947), on the contrary, found that hepatitis γ-globulin was about 10 times as effective as normal γ-globulin in reacting with this reagent. Similar results were obtained with the thymol reagent. A normal γ-globulin electrophoretically prepared, did not give any flocculation after 24 hours but a γ-globulin prepared from hepatitis serum did so. Armas-Cruz, Lobo-Parga *et al.* (1952) who also worked with electrophoretically prepared protein fractions found a higher percentage of positive results with hepatitis γ-globulin than with normal in a series of tests (cephalin-cholesterol reaction, colloidal gold and colloidal red reactions, thymol turbidity test and water dilution test). On the other hand, Albertsen, Christoffersen *et al.* (1950) working with γ-globulin obtained by ethanol precipitation of serum at low temperature found a higher thymol turbidity with normal γ-globulin than with hepatitis γ-globulin. Kunkel and Hoagland (1947) and Armas-Cruz *et al.* (1952) added γ-globulins, isolated from thymol positive cases or from normal cases, to normal sera. These authors discussed the possibility of a difference between these γ-globulins in their ability to give positive thymol values under these conditions.

However, a closer examination of the literature in this field shows somewhat divergent opinions regarding the role played by normal γ-globulin in flocculation reactions. γ-globulin prepared by electrophoresis gave a faint turbidity with the thymol reagent according to Maclagan and Bunn and Armas-Cruz *et al.* and these results are confirmed by Albertsen *et al.* with ethanol-precipitated γ-globulin. Recant, Chargaff and Hanger (1945) and Kunkel and Hoagland (1947) working with γ-globulin obtained by electrophoresis and Bauer (1949) working with ethanol-precipitated γ-globulin obtained no reaction with the thymol reagent. In the cephalin-cholesterol reaction γ-globulin isolated either by electrophoresis or cold precipitation always gave precipitation (Armas-Cruz *et al.*, Bauer). Moore and Pierson (1945), however, found a difference between γ-globulin isolated by electrophoresis at pH 7.4 and 8.6; in the latter case it did not precipitate with the lipid reagent. A new point has been introduced in the discussion by Franklin and Kunkel (1956). According to these authors there is a difference in the ability of different parts of γ-globulin to affect the flocculation reactions. The slow component (as determined by electrophoresis) of γ-globulin is mainly concerned with the thymol and cephalin-cholesterol reactions, and the part of γ-globulin, that has an intermediate mobility (the peak fraction), precipitates in the zinc sulphate reaction. Franklin, Bean *et al.* (1951b) found, however, no statistical correlation between the γ_1-globulin content of serum and the zinc sulphate turbidity. Cases with agammaglobulinemia show a negative zinc sulphate reaction (Good and Varco 1955).

The effect of albumin on flocculation reactions

A report published by Moore, Pierson, Hanger *et al.* in 1945 stated that albumin, isolated by electrophoresis from hepatitis sera, had not the same inhibiting effect on the cephalin-cholesterol reaction as that obtained from normal sera. They conducted tests with electrophoretically isolated γ-globulin whereby normal albumin was shown to have a stronger inhibiting effect on the flocculation of this globulin than the hepatitis albumin. In a work published in 1954, Hanger declared that the stabilising protein factor in the cephalin-cholesterol reaction probably consists of a labile component in the albumin–α_1-globulin complex, which is rich in lipids (cf. also Martin 1948 and Bauer 1949). Maclagan and Bunn in 1947 and Martin in 1949 arrived at similar results with regard to the thymol reaction, e.g. that the stabilising effect of the albumin is reduced in cases of hepatitis. Albertsen and co-workers (1950) have, however, been unable to confirm these results.

According to Maclagan and Bunn (1947) and also according to Bauer (1949), however, the albumin inhibition in the cephalin-cholesterol reaction appears only in an albumin concentration so high that it has no equivalent in serum. In the thymol reaction, also, the isolated albumin, added to positive sera does not exercise its restrictive function until a rather high concentration

is attained (Kunkel and Hoagland 1947, Wunderly and Wuhrmann 1947). In experiments with mixed isolated fractions albumin had an inhibiting effect when used in physiological concentrations according to Maclagan and Bunn (1947), but not in the experiments described by Martin (1948). De la Huerga and Popper (1950) added human serum albumin to γ-globulin, and performed the zinc sulphate reaction with the mixture so obtained. They found that an increase in the concentration of albumin produced first higher, then progressively lower degrees of turbidity, finally reaching a figure beneath the level obtained by the addition of pure γ-globulin only to the reagent. The experimental addition of albumin to whole serum caused a reduction in the reaction. Cases with analbuminemia show positive flocculation reactions (Bennhold 1955).

The effect of α- and β-globulins on flocculation reactions

Reports on the effects of α- and β-globulins are much less frequently encountered in the literature. Maclagan and Bunn found that electrophoretically prepared hepatitis $(\alpha + \beta)$-globulin gave a positive flocculation reaction when mixed with the cephalin-cholesterol reagent, even in the absence of γ-globulin; whereas normal $(\alpha + \beta)$-globulin did not affect it in any way. A qualitative dissimilarity in thymol reactions was observed between these globulins, prepared from various sera, when mixed with γ-globulin; namely, that the hepatitis $(\alpha + \beta)$-globulin produced a stronger reaction than normal $(\alpha + \beta)$-globulin. According to Bauer, the cephalin-cholesterol reagent flocculates isolated β_1-metal combining protein.

This account is solely concerned with the works which have been published on the subject of the cephalin-cholesterol reaction, the thymol reaction and the zinc sulphate reaction. Similar experiments with isolated fractions have been performed with the colloidal gold reaction (Gray 1942, Kabat, Hanger and Moore 1943, Maclagan and Bunn 1947, and Bauer 1949), Weltmann's reaction and the magnesium chloride reaction (Bauer 1949, Knedel and Bube 1955), Takata's reaction, and the sublimate reaction according to Gros (Albertsen and co-workers 1950, Knedel and Bube 1955). In this body of work, the author would like to give particular emphasis to that of Knedel and Bube, in which they demonstrate the markedly inhibitive effect of α_2-globulin. In other respects, their results all concur with the decisive part played by the γ-globulin concentration in all these reactions. But there is no such unanimity concerning the inhibitors of flocculation reactions, and the problem of the "qualitative" dissimilarities between the albumin and γ-globulin derived from normal and those derived from hepatitic sources is also still unsolved.

2. Lipoproteins and glycoproteins

Lipoproteins and flocculation reactions

The decisive role played therein by the lipids is a characteristic feature of the lability reactions. Close study of the chemically oriented section of the literature shows that at a comparatively early stage the importance of the fats in the precipitation of serum protein had been observed. (These works are fully documented by Nikkilä 1953, and Marner 1955.) In the first place, the globulin which precipitates at low ionic strength was found to have a high lipid or phosphorus content (see, for instance, Hardy 1905, Haslam 1913, Sørensen 1923, Frankenthal 1925, etc. etc.). In the second place, it was possible to cause precipitation in serum protein by the addition of fatty emulsions (see, e.g. Chick 1914, and Went and Farago 1931, etc.).

Two interesting types of lability reactions are worthy of mention in this context. In the first, protein precipitation takes place concurrently with that of the fats already present in the serum (thymol reaction type), in the second, the precipitation of the protein is accelerated by the presence of fatty emulsions in the reagent (cephalin-cholesterol reaction type). In the latter type of reaction, it has generally been possible to secure the reaction without regard to the presence of available fat in the serum; in the former, on the other hand, the result of the protein flocculation will be affected by the serum lipids.

Maclagan (1944) observed in analysis of his thymol precipitates a lipid content of 25%, whereas the protein content was 38%. The importance of the lipids in the thymol reaction has been corroborated by strongly affirmative testimony from many other quarters. Cohen and Thompson (1947) and Kunkel and Hoagland (1947) have examined sera by electrophoresis after subjecting them to precipitation with the thymol reagent, and observed that a marked decline in the β-globulin was thereby produced (as much as 50%, whereas the γ-globulin underwent only a 4% decrease). The analysis of the precipitate involves much greater technical difficulties; the former writers regard it as consisting mainly of β-globulin, with a small percentage of α_2-globulin, whereas the latter have perceived a main content of β-globulin and a fast-moving γ-globulin in the precipitate.

An ether extraction of the serum lipids, according to MacFarlane's method, rendered a thymol positive serum negative. "There is no evidence that an abnormal lipid is present in hepatitis serum, since the negative thymol test obtained after ether extraction can be made positive by a variety of lipid preparations (cephalin, crude lecithin, cholesterol) and even by normal serum. Aqueous suspensions of the residues of the ether extracts from normal or hepatitis sera exhibited similar reactivating powers" (Recant, Chargaff and Hanger 1945).

Bauer (1949) has demonstrated that γ-globulin does not produce a

positive thymol reaction until α_1- or β-lipoprotein is added to it. γ-globulin which has been isolated by electrophoresis will not precipitate with the thymol reagent unless lipids are present (Kunkel and Hoagland 1947). According to the same authors, the thymol reaction in the early stages of hepatitis is determined by the lipid content of the serum rather than by the protein variations. The use of the thymol test as a means of ascertaining the lipid content has been suggested by Popper, Steigmann *et al.* (1949). The zinc sulphate test is also, although not so markedly, affected by the serum lipids (Popper and de la Huerga 1950). The significance of the lipids in the "water dilution" test has been indicated by Dreyfuss (1948), Adner (1951) and many others.

Glycoproteins and flocculation reactions

Bergstermann (1954) has conducted analyses of numerous sera, with special reference to the protein-bound carbohydrates, the electrophoretic picture, and Weltmann's reaction. He discovered a remarkable degree of correlation between a shortened Weltmann band, i.e. stabilisation of the serum proteins, and an increased quantity of protein-bound carbohydrate in serum. In these cases the electrophoretic diagrams almost invariably revealed an increase in the α-globulin content (cf. also Olhagen 1947). The experimental addition of a mucoprotein isolated according to Winzler to a serum with a lengthened coagulation band also resulted in a shortening of the band (Bergstermann). The findings published by Poli and co-workers (1951, 1954) also emphasise the part played by the glycoproteins in the end-result of Weltmann's reaction. It is worth noting that the clinical conditions associated with a shortened Weltmann band also produce low values in the thymol reactions (Mawson 1948, Opperman 1950). Anderson, Lockey and Maclagan (1955) have also observed that in cases showing a high mucoprotein level in the serum, the thymol, zinc sulphate and colloidal gold reactions are normal. The experimental addition of a mucoprotein isolated from urine caused a thymol-positive serum to become thymol-negative.

Ducci (1951) maintains that sera from obstructive jaundice contain an inhibiting factor working in the lability reactions. He added commercial γ-globulin firstly to normal sera, and secondly to sera obtained from cases of obstructive jaundice and found that in the second case a larger quantity of γ-globulin was required in order to obtain a positive lability reaction. Brante (1952) has suggested that the inhibiting factor may perhaps be a mucoprotein. Similar observations have been made by Popper (1951) but according to this author the factor is probably of biliary origin (lecithin?)

Summary

The dependence of the lability reactions on albumin and γ-globulin is fairly well established. In this connection, there is, however, some disagree-

ment about the difference reported between normal albumins and γ-globulins and these fractions isolated from cases with lesions of the liver parenchyma. The globulins with intermediate electrophoretic mobility have not been sufficiently investigated for their effect on the lability reactions, possibly because of the difficulty of isolating these proteins. On the other hand, however, some work has been done on lipoproteins and glycoproteins, which are the main constituents of these proteins, and some indirect evidence has been collected, leading to the conclusion that the former act as promotors and the latter as inhibitors of the serum protein precipitation in lability reactions.

B. *Methods 1—7*

(1) *Analytical electrophoresis on paper*

Two methods have been used: (A) an older method in which the paper is placed between two glass slides; this method is of the same type as that described by Köiw, Wallenius and Grönwall in 1952. (B) In a later method the electrophoresis is performed in a damp container with the paper laid over plexiglass needles. The apparatus is a modified version of that described by Dettker and Andurén (1954). This method gives more clearly-defined protein bands and a more distinct separation than the former; this is especially noticeable in the more effective dissolution between albumin and α_1-globulin thereby obtained. Filter paper, type Whatman no. 1, has been used. The photographed strips reproduced on page 30 are electrophoretic runnings on cellulose acetate paper[1] according to Kohn (1956). An approximate quantity of 0.007 ml serum has been applied through a micro-pipette, forming a narrow streak on the paper. The potential applied to the paper in method A is 3.3 V/cm; in method B, 4 V/cm for a duration of 16 hours.

Buffer solution.—Veronal acetate buffer, pH 8.6, ionic strength 0.10, according to Michaelis (1931) has been used. In some later experiments described in part II the electrophoresis has been performed with the same buffer at pH 9.1. The reproduced electrophoresis strips have been run on the buffer described by Aronsson and Grönwall (1957).

Colouring and evaluation of the strips.—After electrophoresis the strips are dried at 100° C for 10 minutes. They are then dyed for 10 minutes in a colour-bath in order to develop the protein spots. The background is bleached in a rinsing medium, with four or five consecutive dippings, each of which lasts for 15 minutes. The electrophoresis pattern has been evaluated by means of direct photometry in the type of photoelectric scanning apparatus described by Köiw in 1953. The readings are obtained here by the use of a red filter. Before reading, the strips are rendered transparent by dipping in an immersion solution (Soalin ALV, Kelab

[1] Oxo Ltd., London.

Co., Stockholm; refractive index 1.480). The light absorption recorded as optical density is plotted on a diagram, the various fractions being separated from each other by a perpendicular line drawn through the lowest point between the fractions. The values of optical density within each fraction are then added and the relative percentage computed. As a rule, the sera have been examined in duplicate, and the electrophoresis which proved most successful from a technical point of view has been evaluated.

Staining solution.—0.5 g Naphtalene Black (Amidoschwarz 10 B, Bayer), 350 ml ethanol (96%), 50 ml glacial acetic acid, 100 ml distilled water. The solution is filtered before use.

Rinsing solution.—The same as above, but excluding Naphtalene Black.

The linearity of the technique used herein has been tested by the experimental addition of lyophilised, isolated fractions (albumin and γ-globulin).[1] In the first instance, the albumin was added to a serum with a low concentration of albumin in concentrations up to 4.3 g %; in the second, γ-globulin was added to serum from a case with a low concentration of γ-globulin to a final concentration of 3.6 g %. The system is linear with all concentrations tested.

The error of the method has been calculated from a series of 20 electrophoreses made simultaneously with identical serum. As is shown in Table 2, the estimation of α_1-globulin is subject to a considerable uncertainty, whereas the analysis of other globulins shows an approximate margin of error of about 10%. The error in the determination of albumin is 6% within the limits of concentrations treated.

There is a remarkable dissimilarity in the results of paper electrophoresis reported from different laboratories. This divergence is not confined to electrophoresis performed by very different methods, with various kinds of apparatus and buffer system or the use of dissimilar colouring methods, but also occurs under apparently identical conditions (cf. Pieper and Molinski 1954). The author's normal values (average

TABLE 2. *The reproducibility of the paper electrophoretic technique (method 1 B), investigated by running the same serum simultaneously on 20 strips.*

	Albumin	Globulin			
		α_1	α_2	β	γ
Mean (relative %)	46.3	6.3	9.5	15.7	22.1
Standard deviation	2.9	1.3	1.1	1.7	2.2
Coefficient of variation (%)	6.3	20.3	11.6	10.5	10.0

[1] The author is indebted to AB Kabi, Stockholm, for furnishing these samples.

TABLE 3. *Normal value (average value) of paper electrophoresis in relative percentage.*

Colouring method: Naphthalene black. Recording by photoelectric scanning apparatus.

	No.	Albumin	Globulin			
			α_1	α_2	β	γ
Björnesjö (method 1 A), 1955 b	30	56.5	4.7	9.6	14.1	15.0
Adner (method 1 B), 1957	50	51.9	5.4	8.5	14.1	20.2
Grassman & Hannig, 1952	25	61.3	4.1	8.1	11.0	15.5
Riva & Martini, 1953	12	65.2	4.1	6.7	9.8	14.1
Castagna & Wewalka, 1954	30	57.9	3.2	8.5	11.4	19.0
Pieper & Molinski, 1954	65	49.9	6.6	8.9	12.7	21.9
Roulet & Trinquier, 1955	50	57.4	4.1	7.6	12.3	18.3
Tizzani (men 20–25 yrs.), 1955	35	56.3	4.3	8.4	12.0	19.2

figures) in relative percentage, with some examples of values taken from the literature, are reproduced in Table 3. The principal disparity, as revealed by this table is found in the concentrations of albumin and γ-globulin.

(2) *Gravimetric determination of total protein content of serum*

Here, the copper sulphate reagent is used according to the method advocated by Phillips and van Slyke (1950). They suggested two correctional formulae for conversion from specific weight (G) to g per 100 ml: for normal plasma $373 \times (G - 1.0070)$ and for pathological plasma $365 \times (G - 1.0065)$. In the experiments to be described the latter formula has been used throughout (i.e. also for the normal group).

METHODS FOR FLOCCULATION REACTIONS

Blood samples were obtained from fasting subjects and were investigated on the day of withdrawal.

(3) *Thymol turbidity reaction*

Method according to Maclagan (1944), pH 7.8, ionic strength 0.01. The turbidity is recorded as optical density. The values cited in Chapter II, D. are those read in 1 cm cuvette at 660 mμ in a photoelectric colorimeter (Beckman spectrophotometer model B); other values were obtained in a Pulfrich Stuphenphotometer (Zeiss) with filter S 66.[1] The error of

[1] If divided by 1.2, these values correspond to the Beckman values.

analysis was computed from duplicate determinations on each sample and amounted to 5.4% (coefficient of variation).[1] The normal mean is 0.04 ± 0.01 with an upper normal limit of 0.12.

(4) *Kunkel's zinc sulphate reaction* (1947)

pH 7.5, ionic strength 0.002. This method has been applied in accordance with the original specifications, the turbidity read as in the thymol reaction. Error of the method amounts to 9.8%, and normal mean lies at 0.15 ± 0.03 with an upper normal limit of 0.32.

(5) *Cephalin-cholesterol reaction*

This was performed according to Hanger (1939).

(6) *Takata-Ara's reaction*

Performed in the modification given by Jezler (1929).

(7) *Water dilution test at varying pH*

This method is a modification of that described in an earlier study (Adner 1951), ionic strength about 0.001, varying pH.

Procedure.—100 ml redistilled water is measured into an Erlenmeyer flask. 0.9 ml buffer (veronal buffer, pH 8.6) is added. The mixture is shaken after which 0.1 ml serum is added. 5 ml of this mixture is poured into each of a series of 7 small glass tubes (or 14 for double tests). The following quantities of 0.005 *N* HCl: 0.30, 0.35, 0.40, 0.44, 0.48, 0.52 ml are then added to the tubes in that order and stirred. Immediately after stirring, the series is subjected to close scrutiny against dark background and the amount of turbidity which has been formed is assessed with the naked eye. Should either the first or the last tube in the series show the highest degree of turbidity, another 5 ml of the serum dilution should be poured into an additional tube, and in the first case 0.25 ml, in the second 0.56 ml hydrochloric acid is added. The amount of turbidity obtained is then measured as optical density in a spectrophotometer, in 1 cm cuvette, 420 mμ, against a water blank, the recordings being read after 30 min. No correctional calculations have been made as the quantities of hydrochloric acid added were so insignificant. The highest degree of turbidity recorded was noted. The amounts of hydrochloric acid indicated herein are selected in order to include a pH interval of from about 7.0 to 5.2. The buffer capacity of this system is, however, so low that a given quantity of acid does not yield a similar pH in every case. It is

[1] Error of the method, σ, signifies hereafter "error of a single determination". The calculation is based on analyses of various (50 or more) duplicate determinations at different levels of concentration, to which the following formula has been applied: $\sigma = \sqrt{\Sigma d^2 / 2N}$ (Dahlberg 1948) with N as number of samples and d as difference between the two analyses of each sample. The coefficient of variation is (%) $\sigma / M \times 100$, taking M as the arithmetical mean of the entire material.

therefore advisable to measure pH in the tube showing the highest degree of turbidity. The error of the method (for the maximum turbidity) lies in the region of 7.8%. The normal mean is 0.04 with an upper normal limit at 0.06.

C. *The changes in the electrophoretic pattern of the serum proteins in liver diseases, and their significance for the flocculation reactions*

The works mentioned above have provided many illustrations of the innumerable variety of factors which can affect the outcome of the flocculation reactions. The author now asks himself the following question: What kind of electrophoretic alterations are actually perceptible in cases of increased lability reactions, and do these fluctuations provide an adequate explanation of the entire problem of these reactions? In order to elucidate this problem, a collocation of material based on investigations of sera from cases of liver disease will be presented here. The ensuing discussion will be especially concerned with the thymol turbidity reaction, as it is usually regarded as the most important test in this field.

Methods and material

Paper electrophoresis was performed according to method 1B and the lability reactions according to methods 3, 5 and 6. The material has beeen classified in four separate categories, as follows:

(1) *Normal cases* (50).—These comprise blood donors from the Blood Bank, University Hospital, Upsala. The group includes 42 men and 8 women aged 20–45. In this group, contrary to groups 2, 3 and 4, the blood samples are not taken from fasting subjects but macroscopically turbid sera have been rejected.

(2) *Cases of acute infective hepatitis* (28).—These cases form a group with a markedly homogeneous aetiology, the result of an epidemic of an unusual type, since the infectious agent was spread by ingested oysters (Lindberg-Broman 1956). The sample of blood obtained during the illness, which showed the highest thymol value was selected for analysis. It may be noted that almost 100% of the cases in this epidemic showed increased thymol value. None of those included herein had a thymol value lower than 0.46.

(3) *Cases of obstructive jaundice* (28).—This material is, from an aetiological point of view, heterogeneous, but the cases have in common a low thymol value (less than 0.08). This group also differs from the others inasmuch as the average age of the patients is comparatively high.

(4) *Cases of cirrhosis of the liver* (23).—This material is also heterogeneous, comprising post-hepatitic cirrhoses, alcoholic cirrhoses, and one

case with indications of biliary cirrhosis. It also includes some cases of juvenile cirrhosis, 7–17 years of age. The results presented here consist of samples taken at random without regard to the clinical condition of the subject.

Groups 2, 3 and 4 are tabulated in the appendix. The paper electrophoresis strips reproduced correspond to cases number 1, 2 and 3. (Table V.)

Results

Average figures with the distribution for groups 1, 2, 3, and 4 are cited in Table 4 (cf. also Table 1). A graphical presentation of the material is shown in Fig. 1. The hepatitic group shows a marked divergence from the normal group as regards the concentration of albumin and γ-globulin. There is a perceptible tendency to decrease of α_2-globulin, but the significance of this difference is comparatively slight ($0.05 < p < 0.10$). The β-globulin content is normal. Only two of the 28 cases, i.e. 7%, show figures neither above nor below the normal standard with respect to *all* electrophoretic fractions. But a comparison between groups 2 and 3, which are the most important categories with regard to the problem here presented, yields no evidence of variations in the albumin concentration. Both these groups show an increase of γ-globulin, the hepatitic being slightly higher than the other. The most striking difference between the two groups is, however, the remarkable augmentation of α_1- and α_2-globulin, which takes place in the obstructive jaundice group. Nor does this group 3 show any β-globulin increase when compared with the normal material. The differences between groups 2 and 3 which can be seen in this figure are statistically presented in Table 5. The $(\alpha_1 + \alpha_2)/\gamma$ quotients for both groups are also calculated in this table.

Table 4. *The distribution of the electrophoretic serum protein fractions in the author's material.*

Paper electrophoresis with method 1 B. The values (mean and standard deviation of the groups) are given in g per 100 ml.

Group	No.	Albumin	Globulin			
			α_1	α_2	β	γ
Normal	50	3.71±0.27	0.38±0.08	0.61±0.10	1.01±0.16	1.45±0.25
Hepatitis	28	2.93±0.32	0.40±0.13	0.56±0.12	0.97±0.19	2.14±0.43
Obstructive jaundice	28	2.91±0.51	0.60±0.10	0.94±0.19	1.05±0.20	1.68±0.27
Liver cirrhosis	23	2.79±0.65	0.35±0.09	0.63±0.12	0.93±0.30	2.80±1.09[a]

[a] Median = 2.53.

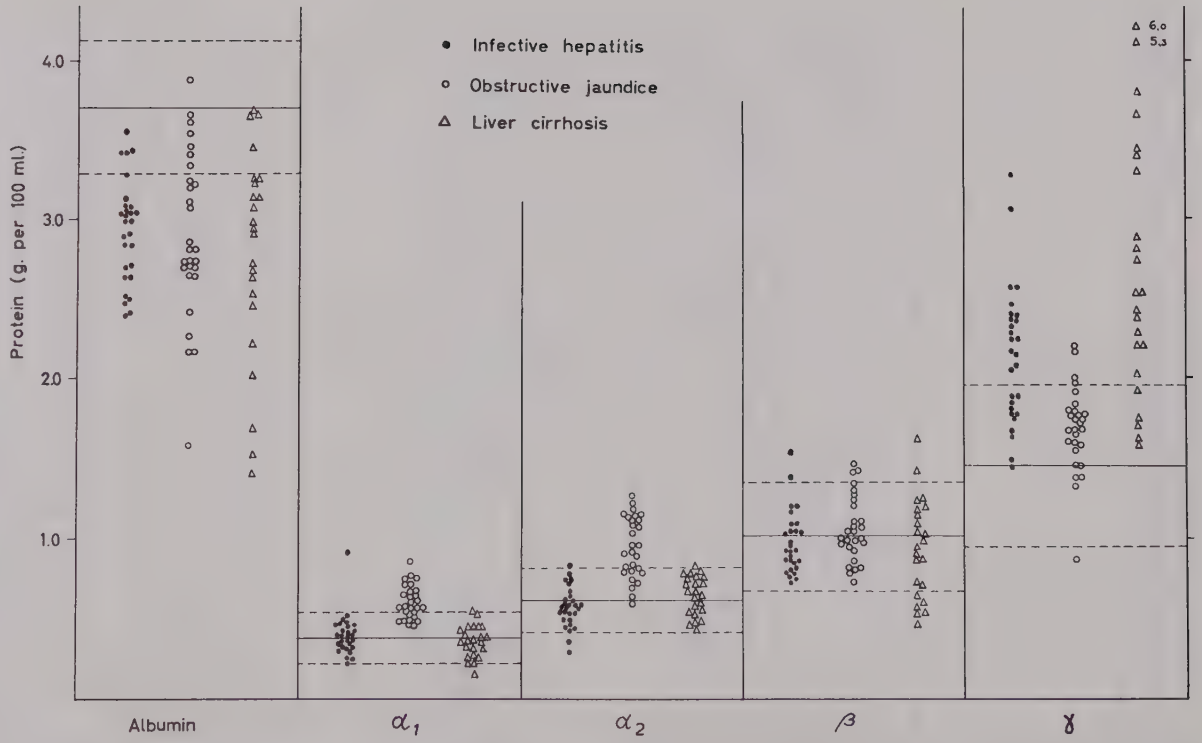

Fig. 1. Diagram showing a comparison between the electrophoretic patterns of serum proteins in acute infective hepatitis, obstructive jaundice and liver cirrhosis. The normal range within $\pm 2\sigma$ is given (——, mean; - - - -, normal limits).

TABLE 5. *A statistical analysis of the pertinent data in the two groups hepatitis and obstructive jaundice.*

H = infective hepatitis; OJ = obstructive jaundice; t = (difference between means)/(standard error of the difference of the means); p = level of significance. Statistical calculations according to Croxton (1953).

	α_1-globulin g/100 ml		α_2-globulin g/100 ml		γ-globulin g/100 ml		$\frac{\alpha_1+\alpha_2}{\gamma\text{-globulin}}$	
	H	OJ	H	OJ	H	OJ	H	OJ
Mean ($N=28$)	0.395	0.600	0.565	0.940	2.139	1.676	0.443	0.952
Standard error of mean	0.0239	0.0189	0.0231	0.0360	0.0821	0.0422	0.0252	0.0535
Difference between means		0.205		0.375	0.463			0.509
t		6.7		8.8	5.0			8.6
p		<0.001		<0.001	<0.001			<0.001

It will thus be seen, that, although quantitatively unimportant, the difference between groups 2 and 3 in these fractions is extremely significant from a statistical point of view.

The cirrhosis group differs, as it would be expected to do, from the hepatitis group with regard to albumin and γ-globulin (Fig. 1). In connection with the normal group, it should be specially noted that the β-globulin content is, if anything, slightly decreased ($0.10 < p < 0.20$). A tendency to low α_1-globulin may perhaps be discerned but the difference is not significant ($0.10 < p < 0.20$). In the cirrhosis group, the average of the $(\alpha_1 + \alpha_2)/\gamma$ ratio was 0.365, i.e. still lower than in the hepatitis group. The variation in the thymol reactions of these groups is, however, given special prominence in this study. The thymol values are shown in conjunction with the γ-globulin concentration in Fig. 2. The thymol values of the hepatitic group as reproduced in this figure are thus seen to be usually somewhat higher than those of the other group. In Fig. 3. another flocculation reaction is shown, namely the cephalin-cholesterol reaction, in a similar context. The same tendency is revealed, although the modification used herein is ill-adapted to quantitative investigation. The zinc sulphate reaction was not analysed in this series; the Takata reaction has been used instead. This reaction can only be said to show a high positive frequency (74%) among the liver cirrhosis patients, and a low positive frequency (26%) among the cases of hepatitis, so it may perhaps have provided a more accurate estimate of increased γ-globulin concentration in this material than the other reactions investigated.

The significance of the changes in α_1- and α_2-globulin described above is given additional emphasis by the four electrophoresis strips reproduced

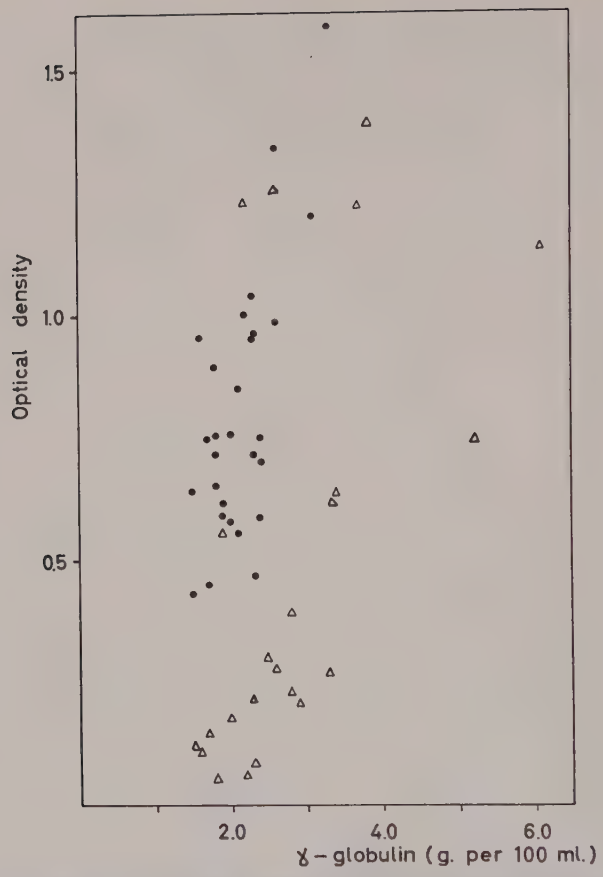

Fig. 2. Graph showing the relationship between the thymol turbidity test and the γ-globulin content of serum in a material consisting of acute infective hepatitis and liver cirrhosis. •, infective hepatitis; △, liver cirrhosis.

in Fig. 4. Attention is especially drawn to the almost total absence of α_1-globulin and the faster moving component of α_2-globulin in the cirrhosis of the liver. The difference between cholecystitis and hepatitis is clearly indicated; relatively the faster-moving component of the α_2-globulin appears to be markedly increased in cholecystitis.

Discussion

The electrophoresis results presented tally very well with those shown in earlier investigations (cf. especially Owen and Robertson 1956), although the material has been selected in accordance with rather different

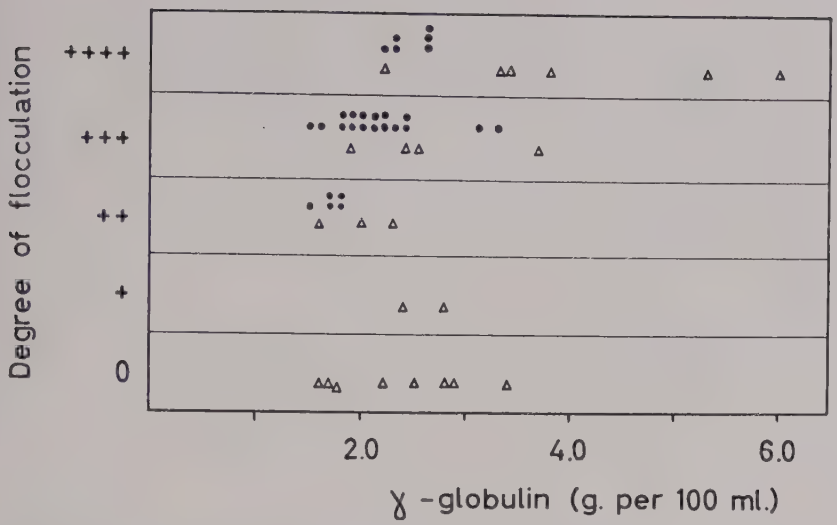

Fig. 3. A comparison between the hepatitis and the liver cirrhosis groups with reference to the cephalin-cholesterol reaction and the γ-globulin content of serum. ●, infective hepatitis; △, liver cirrhosis.

o	no change in the solution
+	a slight sediment, no clearing of the supernatant
++	a slight sediment and a slight clearing of the supernatant
+++	heavy sediment and incomplete clearing of the supernatant
++++	heavy sediment and complete clearing of the supernatant.

principles. This investigation has revealed no increase of β-globulins in any of the groups examined, and it calls for re-emphasis. This is different from the results of, e.g., Popper, Bean *et al.* in 1951 (moving boundary electrophoresis) with whose results it is otherwise very compatible.

Albumin and γ-globulin are, of course, those fractions most frequently discussed in connection with the flocculation reactions. These two proteins clearly differentiate the group with liver parenchyma lesions from the normal group. It is, however, necessary to point out that the obstructive jaundice group shows divergencies from normality in these fractions in the same direction as those of the hepatitis group; not very marked variations in the γ-globulin but for albumin quite as clearly defined as those of the hepatitis group. The proportions of α_1- and α_2-globulin must therefore be closely studied, with regard to both the problems of differential diagnosis (cf. Greenspan and Dreiling 1957) and in the discussion of the part played by the serum proteins in the lability reactions.

A comparison between the hepatitis and the cirrhosis groups reveals the fact that sera with identical γ-globulin concentration can show highly

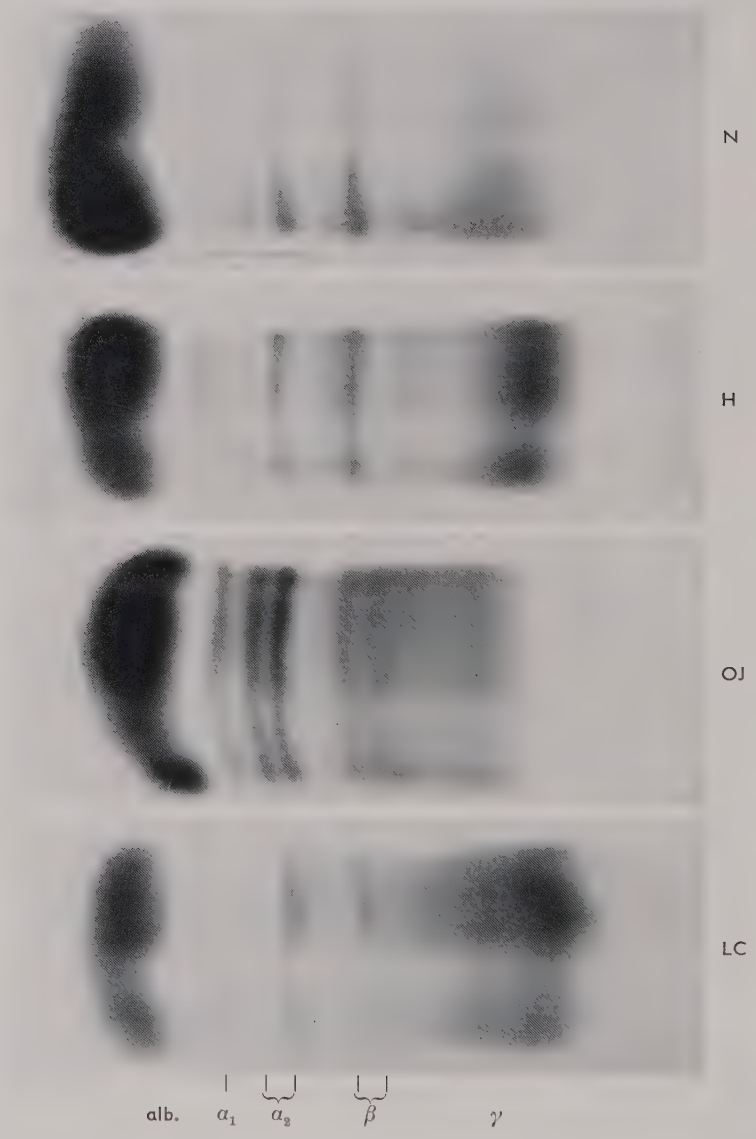

Fig. 4. Electrophoresis on paper (cellulose acetate) of sera from cases with different liver diseases. Buffer according to Aronsson and Grönwall 1957. N, normal case; H, infective hepatitis; OJ, cholecystitis; LC, liver cirrhosis.

divergent thymol values. The correlation of the thymol reaction and the γ-globulin concentration will be treated more comprehensively in a following section. If the albumin were the decisive factor, the thymol value should then be higher in the cirrhosis group. The same correspondence between the thymol reaction and γ-globulin can be deduced from Brante's data (1952). In the author's opinion this divergence between the two groups may possibly be ascribed to quantitative and qualitative differences in the serum lipids. Reference might also be made to the work of Kunkel and Hoagland (1947), in which they state that the thymol reaction in the initial stages of acute hepatitis appears to be determined by the level of the lipids in the serum, rather than by that of the γ-globulin. According to Steigmann, Popper *et al.* (1949) the cephalin-cholesterol flocculation and thymol turbidity were increased in a higher percentage in infective hepatitis, whereas the zinc sulphate and Takata-Ara tests were more frequently positive in liver cirrhosis.

The quotient $(\alpha_1 + \alpha_2)/\gamma$ gives in a single value a synthesis of the material, but otherwise the difference between the hepatitis and obstructive jaundice groups is not more clearly defined by this quotient than by determining α_2-globulin alone. This quotient is, however, fairly important from the chemical point of view, inasmuch as it represents the proportional relation between the serum globulins with the greatest and those with the smallest carbohydrate content. A more detailed discussion of a physico-chemical aspect of this quotient will be found on page 78.

The data presented above are recapitulated in Table 6, in the final column of which some of the factors, discussed above or in the earlier bibliography, have been included.

TABLE 6. *A summary of the most important data of chapter II C.*

Groups to discriminate	Protein changes found	Other changes assumed for the unexplained high thymol values in hepatitis
Hepatitis from normal	albumin decreased, γ-globulin increased	"Qualitative" changes of albumin and/or γ-globulin?
Hepatitis from obstructive jaundice	no increase of α_1- and α_2-globulins (slightly increased γ-globulin level)	"Inhibiting" substances in OJ? Differences in lipid composition?
Hepatitis from cirrhosis	only moderate decrease of albumin and slight increase of γ-globulin	Lipid content? and composition?

Finally, it must of course be emphatically stated that serum electrophoresis, performed under present-day conditions, places a somewhat imperfect tool in the hands of the investigator, as it provides only a rather rough-and-ready fractioning of the serum protein. The author is also fully conscious of the fact that a discussion of these problems cannot be entered into without a minute analysis of the lipoproteins (in the category of the α_1-, α_2- and β-globulins). Additional enlightenment on the subjects alluded to here might perhaps be obtained from a lipoprotein electrophoresis, but on the other hand, the protein component, in its capacity as a carrier of the lipids, is almost certainly the decisive factor. An attempt will be made in the following study (part II) to determine the probable importance of the lipoproteins in this connection, as revealed by later findings.

D. *The statistical correlation between some flocculation reactions and the electrophoretic pattern of the serum proteins*

In an earlier investigation (1951) the author gave some preliminary data on the protein precipitation (euglobulin in the nomenclature of Sørensen) obtained when serum is diluted into water at varying pH.

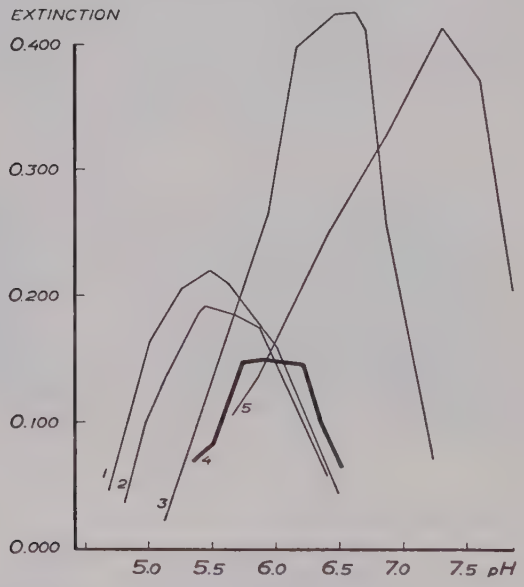

Fig. 5. Some curves obtained by the euglobulin precipitation test. *1*, Cardiac infarction; *2*, choledocholithiasis; *3*, liver cirrhosis; *4*, one normal case; *5*, γ-myeloma. (Adner 1951.)

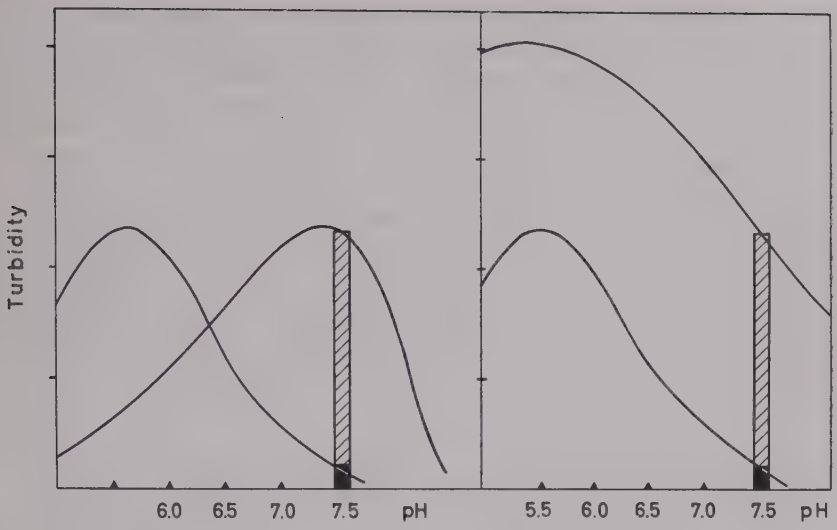

Fig. 6. Graph illustrating how the same result of a flocculation reaction, performed at a single neutral pH, may correspond to precipitation curves with different pH's of maximum turbidity. In the left graph the normal precipitation curve and a curve displaced towards alkalinity are sketched; in the right graph a precipitation curve of a high maximal turbidity with its optimal pH at the normal position is superimposed on the normal curve. Black piles, normal values; striated piles, pathologically increased value of the flocculation reaction.

This "water dilution test" is a modification of a routine test, called by a great variety of names (for further information see Heepe's review, 1953). Other workers who have used this test combined with varying pH are Wolff (1938), Boselli (1948), Vargues (1952) and Ménaché (1955).[1]

The author's observations may be recapitulated under the following points; similar conclusions are found in the papers by Vargues (1955) and Simon (1956).

1. The protein precipitation was recorded as turbidity (optical density). When the turbidity values were plotted on a diagram against pH a curve was obtained with a maximum over a certain pH of maximum turbidity (optimal pH, pH of minimum stability). From 5.8, the normal average, this pH shifted in both directions in different pathological conditions. In some cases, and first and foremost in liver parenchyma diseases, it was displaced in an alkaline direction; in other cases, on the contrary, into the acid region, as for instance in some acute infectious diseases, myocardial infarction etc. (Fig. 5, taken from Adner 1951).

[1] The observation on the turbidity developed by serum when diluted into water must be an old one, but the first investigator who attempted a quantitative evaluation of the phenomenon was Panum (1851 and 1852). The variations in the yield of precipitation by the presence of acid were also well recognized by that author.

2. The turbidity value at the pH of maximum turbidity also varied from case to case; it could, however, be shown to be correlated with the total amount of globulins present. A few exceptions from this rule were recorded, namely, one case of diabetes with lipemia and some cases of myeloma.

3. Finally it was observed that those cases with a pH of maximum turbidity displaced in the alkaline direction also gave positive results in the ordinary flocculation reactions (Table 1 in the paper previously referred to). However, a serum with a pH of maximum turbidity displaced into the acid direction could sometimes display positivity in the lability reactions, but only if the turbidity at this pH were sufficiently high. In Fig. 6 a schematic picture of this point is given, i.e. the relationship between the euglobulin precipitation curve studied over a wide pH range and a single dilution test performed at one neutral pH. Attention is called to the fact that the thymol and zinc sulphate reactions which are more closely investigated below are both performed in this region of pH (pH 7.8 and 7.5 respectively). The position of the pH of maximum turbidity can be considered as being a result of the interplay between the serum proteins with an isoelectric point in the acid region and those with an alkaline or neutral isoelectric point. This topic will be extensively discussed in part II in the chapter on protein-protein interactions.

In this section the maximum euglobulin turbidity will be further studied with an enlarged material, and the object has been to find the numerical relationship to the electrophoretic serum protein fractions. From the same point of view two ordinary flocculation reactions have also been studied namely the thymol and zinc sulphate tests.

Methods and material

Paper electrophoresis is performed according to method 1 A, and the flocculation reactions according to methods 3, 4 and 7. The coefficients of correlation are calculated between the turbidities obtained in the flocculation reactions and the concentrations of the main electrophoretic serum protein fractions (Croxton 1953) assuming a simple linear correlation.

The samples examined have been obtained from a mixed hospital material, consisting of 62 cases of fairly common pathological conditions. The cases have been selected, as the erythrocyte sedimentation rate was increased in all cases. 10 cases could be characterised as liver parenchyma disease. No cases of myelomatosis are included, since this disease so very often gives quite irregular and atypical results in the lability reactions (Maclagan, Martin and Lunnon 1952, Sonnet 1953, and Brante 1956). The composition of the material is tabulated in Appendix, Table IV. In addition four normal cases (blood donors) are included.

TABLE 7. *The coefficients of correlation between three flocculation reactions and the main serum protein fractions.*

Paper electrophoresis according to method 1 A. A coefficient of correlation $> +0.2$ or < -0.2 deviates significantly from 0. Total globulin includes α_1-globulin.

	Albumin	Globulin				Total globulin
		α_1	α_2	β	γ	
Thymol reaction	−0.15	−0.17	−0.20	−0.21	+0.62	+0.34
Zinc sulphate reaction	−0.28	−0.02	−0.24	−0.03	+0.88	+0.62
Water dilution test at varying pH	−0.49	+0.11	+0.28	+0.33	+0.67	+0.77

RESULTS

The coefficients of correlation are given in Table 7. In Figs. 7, 8 and 9 a graphical representation is given of the relationship between the results of the reactions and the concentration of γ-globulin and total globulin

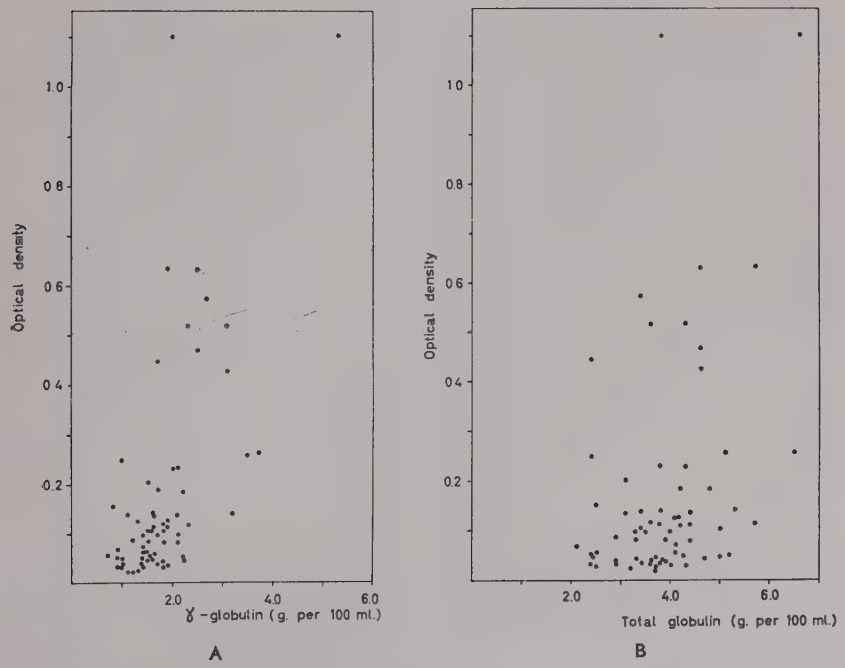

Fig. 7. Graph showing the relationship between the thymol turbidity test and (A) the γ-globulin content of serum, (B) the total globulin content of serum.

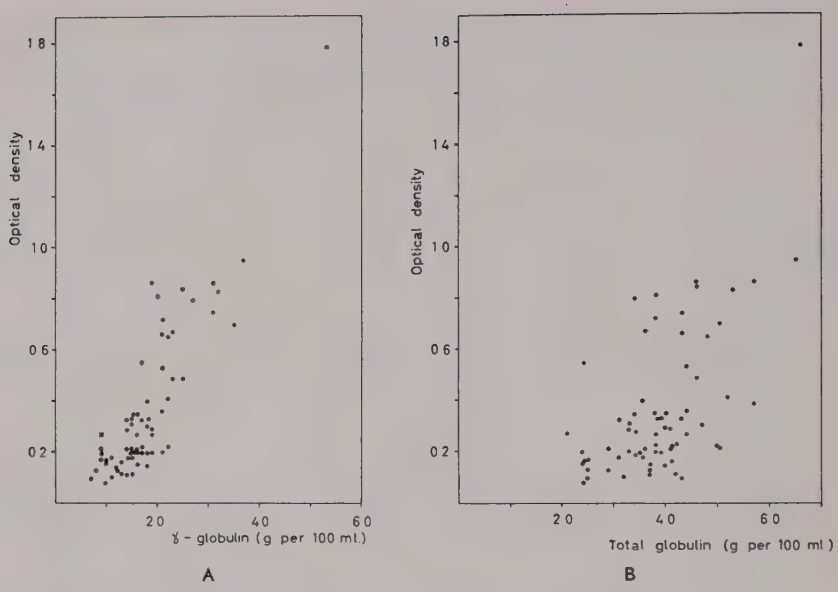

Fig. 8. Diagram showing the relationship between the zinc sulphate test and (A) the γ-globulin content of serum, (B) the total globulin content of serum.

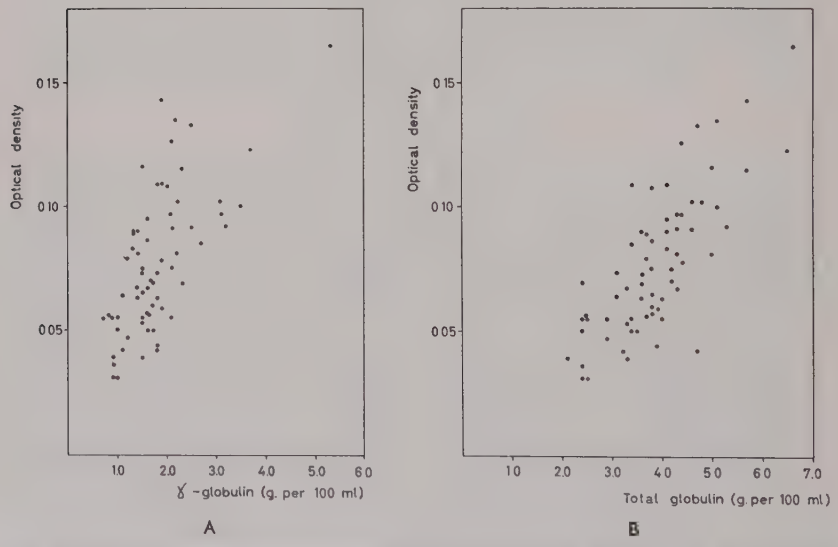

Fig. 9. Diagram showing the relationship between the "water dilution test with varying pH" and (A) the γ-globulin content of serum, (B) the total globulin content of serum.

(including α_1-globulin). A numerical value of the coefficient of correlation exceeding 0.50 is considered as indicating a fairly good correlation.

The dominating role played by γ-globulin in all tests is clearly shown, the coefficient of correlation reaching its highest value, 0.88, in the zinc sulphate reaction, compared with 0.62 in the thymol test. As will be shown later, the assumption of a linear correlation cannot, however, be maintained; at least not in the thymol reaction. If the thymol values are plotted against the square of the concentrations of γ-globulin a coefficient of correlation as high as 0.80 will be obtained. It is worth noting that all other protein fractions are negatively correlated to the thymol and zinc sulphate reactions, even though the correlation is very low. The euglobulin turbidity is positively correlated to all globulin fractions, but principally to γ-globulin (coefficient of correlation 0.67). The dependence of this reaction on all globulins is also reflected in the high correlation to the total globulin (0.77). In the zinc sulphate test the influence of the total globulin is also noticeable, but in the thymol test it is practically imperceptible. The relationship to albumin will be further discussed below.

Discussion

The coefficient of correlation 0.88 between the zinc sulphate test and the γ-globulin concentration is the highest reported in the literature. De la Huerga and Popper (1950) calculated the correlation coefficient to 0.67 in a material of cases, among which were found several myelomas. Hainline, Wilson *et al.* (1956) found in a material, the composition of which is not given, a coefficient of correlation of 0.59. These investigations were accomplished with moving boundary electrophoresis. It has to be pointed out that in all these studies on the connection between the lability reactions and electrophoretic protein fractions, the coefficients found will vary with the composition of the material (cf. also Fig. 2). In particular the aberrant results of the tests in cases of myeloma must again be emphasised.

The relatively poor correlation between the thymol test and the γ-globulin concentration is confirmed. It has previously been explained by the great sensitivity of this reaction to variations of the serum lipids. The author wishes to lay stress upon two points in the construction of the thymol test and the zinc sulphate test. The thymol test is performed at a higher ionic strength and, as will be shown later on, the lipid carrying proteins will precipitate earlier than the γ-globulin when the ionic strength is decreased. The thymol test is also performed at a pH somewhat more alkaline than the zinc sulphate test. The inhibiting effect of the other globulins and albumin on the γ-globulin precipitation will accordingly be more pronounced. This particular point of view will be further elucidated in the discussion on protein-protein interactions

(part II, chapter IV). In addition, the deviation from linearity in this correlation is quite evident in the thymol test. The particular ionic effect of zinc on the protein precipitation will not be discussed in this paper, although it is certainly of some significance.

The negative correlation between albumin and the different tests is most pronounced in the euglobulin turbidity reaction (coefficient of correlation -0.49). The reason for this is not quite clear, but a clue may be provided by the fact that the precipitation is here performed at the optimal pH. The correlation between the thymol and zinc sulphate tests and albumin is very low indeed, and on about the same level as α_2-globulin. This is not in accordance with the important role as an inhibitor assigned to albumin in these tests. The inhibiting effect of albumin shows some peculiar aspects which will be further demonstrated in the experiments on protein-protein interactions.

Although the correlation with α_1-, α_2- and γ-globulin is low, the difference in signs of the coefficients of correlation in the thymol test and zinc sulphate test on the one hand, and the water dilution test on the other, agrees well with the results obtained with mixtures of isolated protein fractions (part II, chapter IV). The main difference between these two groups of tests is the pH of the procedure. In a test working at a pH slightly alkaline to the pH of minimum stability of γ-globulin (part of) each other globulin displays an inhibiting effect, whereas in a test at a pH slightly below neutrality the other globulins coprecipitate with γ-globulin. The correlation between the tests and the total globulin is also connected with this effect of pH. The close correlation between the zinc sulphate test and the total globulin was already recognised by Kunkel (1947). In Hainline's and Wilson's material this coefficient of correlation was calculated to 0.39, and by Maclagan, Martin and Lunnon (1952) to 0.60 as opposed to 0.23 for the correlation between the thymol test and the total globulin. However, the globulins were estimated by these authors using precipitation methods.

The question is still further complicated by the fact that the lipid-carrying proteins may act as promotors and inhibitors of precipitation at the same time, at least when the precipitation is evaluated by turbidimetry (part II, chapter IV). The variable modifying effect on the precipitation of γ-globulin exerted by the lipoproteins and also by the glycoproteins thus seems to constitute an essential distinction between the different flocculation tests.

Finally, attention is called to the existence of some co-variation between the different electrophoretic serum protein fractions. Riva (1952) demonstrated a negative correlation between α_2-globulin and γ-globulin. A positive correlation between α_1- and α_2-globulin has been observed by Popper, Bean and de la Huerga (1951) and between α_1-, α_2- and β-globulin by Sonnet (1956). In the material here presented the negative coefficient of correlation between albumin and γ-globulin

amounted to no more than 0.43. A flocculation test with a high correlation to γ-globulin will consequently also be to a slight extent negatively correlated to albumin. The correlation between γ-globulin and other globulins was still less appreciable.

E. *The effect on some flocculation reactions of experimental addition of isolated protein fractions to whole serum*

In the light of the results given above the importance of the inhibiting effect of albumin might on the whole be questioned. Yet the evidence given in the literature on this point is rather convincing, if at the same time not quite unanimous (see page 16). If the outcome of the flocculation reactions is regarded only as the result of the interplay between albumin and γ-globulin, the experimental addition of these proteins in an isolated state to different pathological sera should lead to a restoration of normal values. In the experiments to be described below, two sera were selected with very low concentrations of albumin and γ-globulin respectively.

Material, methods and results

The two sera investigated were taken from one case of liver cirrhosis (LC, case 4) and one case of agammaglobulinemia (AG, case 5). The concentrations of the different serum protein fractions are presented in Table 8. Paper electrophoresis was performed with method 1 B and total protein determined with method 2. The isolated protein fractions, albumin and γ-globulin (Kabi AB, Stockholm), were prepared by precipitation with ethanol at low temperature (Cohn's method 10) and supplied in a lyophilised state. Varying amounts of the proteins were weighed out and dissolved into the sera, and the thymol test (method 3) and the zinc sulphate test (method 4) were carried out on these mixtures.

Figs. 10 and 11 give the results of these experiments. The accelerating

Table 8. *An electrophoretic analysis of the sera used in chapter II E.*

The values are given in g/100 ml. Values within brackets are relative percentage. AG = agammaglobulinemia; LC = liver cirrhosis.

Serum	Albumin	Globulin				Total protein
		α_1	α_2	β	γ	
AG	3.1 (56)	0.3 (6)	1.0 (18)	1.0 (18)	0.1 (2)	5.5
LC	1.6 (32)	0.2 (3)	0.4 (8)	0.7 (14)	2.1 (43)	5.0

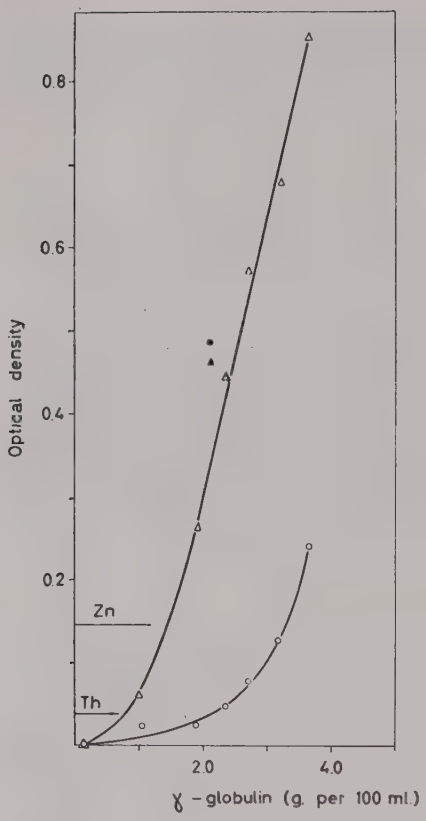

Fig. 10.

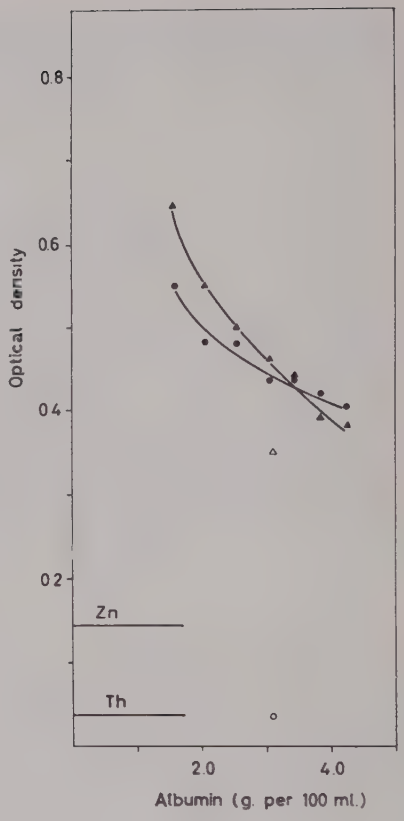

Fig. 11.

Fig. 10. The effect on the flocculation reactions of addition of γ-globulin to a serum from a case of agammaglobulinemia. △, zinc sulphate test; ○, thymol test; ▲ and ●, values obtained by interpolation from Fig. 11, — normal mean of each test.

Fig. 11. The effect on the flocculation reactions of addition of albumin to a serum from a case of liver cirrhosis. ▲, zinc sulphate test; ●, thymol test. △ and ○ values obtained by interpolation from Fig. 10.

effect of γ-globulin on the protein precipitation in the two reactions is clearly shown, but is more pronounced in the presence of the zinc sulphate reagent. The gradual initial slope of the curve with increasing γ-globulin concentration is highly impressive. At low γ-globulin concentrations the relation between turbidity and added globulin deviates from linearity, and this tendency is more pronounced in the thymol reaction. An addition of γ-globulin up to normal concentration levels corresponds to results

of both these lability tests within the normal range. Finally it may be mentioned that this isolated γ-globulin if investigated alone gave a distinct turbidity with the zinc sulphate reagent but none with the thymol reagent (in concentrations up to 2.5 g %).

The inhibiting effect of albumin on the protein precipitation is also distinctly demonstrated. Attention is called to the fact that an albumin/γ-globulin ratio in the neighbourhood of 2[1] forces the values of both tests down to about 0.39. By extrapolation it may be concluded that, in the thymol test at least, only a very high concentration of albumin can suffice to counterbalance the effect of the precipitating proteins so as to obtain a normal value of the test.

If, in addition, those points on the curves are looked at, where the two artificial sera are similar with respect to the concentration of albumin and γ-globulin, some indirect information can be obtained (Figs. 10 and 11, interpolated values). On the one hand, the addition of albumin to serum LC did not suffice to inhibit the protein precipitation down to the level of serum AG. On the other hand, the accelerating effect of γ-globulin on the precipitation in serum AG did not reach the level exhibited by serum LC.

Discussion

These results thus confirm the well-established effects of γ-globulin and albumin. The prompt effect of γ-globulin in the zinc sulphate reaction agrees well with the high coefficient of correlation previously found. In the thymol reaction, it appears as though some inhibitor first had to be overcome before γ-globulin could display its full effect, resulting in the non-linear response. The effect of albumin is convincing, but here again the thymol test in particular forms an exception. The results are in good accordance with those obtained by earlier investigators (p. 16). Two hypotheses may consequently be discussed in order to explain the findings in the experiment with albumin. Firstly, normal serum may contain an inhibitor of precipitation, which is deficient in cirrhosis. Secondly, the cirrhosis serum contains a factor capable of enhancing the precipitation and which is absent in normal serum. As will be shown later on, both theories are probably correct.

The difference between the values found in one experiment and those interpolated from the other experiment may, of course, be explained by assuming, in liver cirrhosis, the existence of a γ-globulin and/or an albumin with qualities deviating from normality, as has been suggested in the literature (p. 15 and p. 16). However, another explanation may also be possible. As is shown in Table 8 the agammaglobulinemia serum has a total concentration of the α_1-, α_2- and β-globulins exceeding the

[1] The author's normal value: 2.6.

serum from the liver cirrhosis with 1 g %. In the following, the theory is put forward that these globulins also may participate in the inhibition of the protein precipitation in the lability tests. These experiments are in some respects comparable with those published by Ducci and already referred to on p. 19. The inhibiting factor in his cases of obstructive jaundice may quite as well be the α-globulins.

Part II

SOME PHYSICO-CHEMICAL ASPECTS ON THE FLOCCULATION REACTIONS WITH SPECIAL EMPHASIS ON THE INTERACTION BETWEEN SERUM PROTEINS

In part I it was stated that quantitative changes in albumin and γ-globulin content do not alone suffice to furnish a complete explanation of the mechanism of flocculation reactions, but that the globulin fractions which move at an intermediary rate in electrophoresis (α_1-, α_2- and β-globulin) must be accredited with some significance in this connection. There is, however, no simple correlation between the concentrations of the electrophoretic protein components and the result of the lability reactions. The entire problem is closely interconnected with the solubility of the serum proteins (exhaustively discussed in Cohn and Edsall 1943). In flocculation reactions the object of study is the solid (precipitated) phase, which supervenes after the balance of solubility has been attained; but the classical experiments in solubility were concerned with the dissolved phase. The most important parameters for the definition of the solubility of the proteins in water are the pH, the albumin concentration, the ionic strength, and the temperature. The fact that every alteration in these parameters causes protein precipitation, especially when additional precipitating agents, such as certain metal ions or other colloids have been introduced, has thus been fully confirmed. In the case of the lability reactions, several such precipitant operations are usually combined with each other. Two possibilities can now be seen, viz. each serum protein takes part in the precipitation in accordance with its defined solubility, and the only factor in any particular case (all other parameters being constant) finally determining the precipitation of this protein is the extent of its concentration. Otherwise the solubility of this protein is modified by the presence of the other proteins, i.e. by protein-protein interaction.

The author has therefore decided to study the precipitation of isolated serum proteins in model experiments adapted thereto, by means of variations in the basic parameters described above, with the exception of temperature as most lability reactions are performed at room temperature. The significance of interaction in connection with the solubility of the various components can be computed by subsequent blending of the isolated proteins in pairs.

Chapter I. Methods

Methods 8—15[1]

Method 8. Preparatory electrophoresis

A. *In filter paper as stabilising medium*

This method was originally proposed by Kunkel and Tiselius (1951) and has been modified by Latner, Raine and Ross (1952), Hartmann and Müller (1952), Swahn (1953) and Laurell (1955). The separation is performed in a bundle of filter papers, "sandwiched" on top of each other.

The electrophoresis "cell" consists of two heavy glass plates (mirror glass, 16 cm × 48 cm) between which the package of papers is placed. The dimensions of the paper (Munktell no. 20/150) were 70 × 12.5 cm and 12 such strips were used in each experiment. The papers are wrapped in an envelope of plastic sheeting which is introduced with the ends of the papers into the buffer vessels. A rectangular piece (size 2 × 8 cm) is cut out on each filter paper strip at a distance of 16 cm from the cathodic end of the paper. The paper bundle is then wrapped up in the plastic sheeting, the anodic edge is dipped into the buffer solution, and the bundle is left lying between the glass plates. Thus the paper bundle is uniformly steeped in buffer and the impurities of the paper are washed away. The upper glass plate is then taken away and the envelope is unwrapped. A paper cube is formed by the small pieces earlier excised from the filter strips, and impregnated with serum. Usually, the serum has not already been dialysed. In the dimensions of the papers described above, 5.5 ml are readily separated. The moistened lump of paper is tightly pressed down into the original aperture of the paper bundle. When the plastic cover encloses it and the glass plates are affixed, the "cell" is ready for electrophoresis. A potential of 400 V (about 5 V/cm) is then applied. The separation is performed at room temperature and run for about 22 hours. The temperature in the cell during electrophoresis is never permitted to rise above 30°C. The separation of the serum proteins may easily be followed by lighting up the cell from below. The yellow albumin and the red β-globulin spot, stained by the small amount of hemoglobin and other pigments present, will then stand out distinctly.

After the electrophoresis the paper bundle is taken out and divided into sections, according to one of the following procedures:

(I) The lowest paper in the bundle, in which no window is cut out, is stained according to the procedure described in method 1 part I. The contours of the main protein spots are copied on to an ordinary paper and this one is laid on the wet paper bundle and the outlines of the spots

[1] Methods 1–7 are described in part I, pp. 20 ff.

are punched on to the paper bundle by means of an awl. After that the bundle is cut according to the main protein spots.

(II) The paper bundle is divided into equally sized cross-segments, 3 cm wide.

The cutting is performed with the aid of a sharp knife and a mallet. The protein solutions are then expressed from the paper segments by centrifuging (2000 r.p.m. for 5 minutes) in specially constructed Perspex tubes, perforated in the bottom and fitting into ordinary 50 ml centrifuge tubes. From these eluates small amounts are taken for protein determination (method 10).

Comments on the procedure described.—It could be shown, by using a serum strongly hemolysed and in addition stained with bromophenolblue, that the procedure including the putting on the glass plates and the cutting of the paper bundle, did not significantly disturb the protein separation. In one run every second paper was stained with Naphtalene black, but no significant difference was found in the protein pattern between different levels in the paper bundle. The expression of the protein buffer solution as described above gives a yield of 70–80 % of all moisture, and the protein recovery is of the same magnitude. In this study no attempt has been made to obtain 100% recovery, and the method has the great advantage of being rapid and giving rather a high protein content of the fractions (more than 0.15 g % for the globulins and about 1.5 g % for albumin as a rule). This fact is important because the separated fractions are very unstable (this applies especially to the globulins), and every delay after separation is accompanied by the formation of a precipitate in the protein solution (cf. also Ott 1956). It was therefore considered as very important to attain a technique which made the protein fractions available for immediate use. It may be added that this deterioration also occurs when the fractions are stored in a refrigerator or kept frozen at -20°. Lyophilisation has been tried, but the lipid-carrying globulins do not redissolve completely afterwards. The intermediate fractions (α_2-, β- and γ_1-globulins) often show a slight turbidity already, when freshly prepared. The significance of this turbidity will be discussed later on (Chapter III).

It is, however, necessary to emphasise the fact that even this form of separation is a comparatively crude procedure as Schultze and Biel (1955) have already stated. Some degree of contamination from contiguous fractions is unavoidable. Fig. 12 shows an experiment in which the isolated protein fractions have been subjected to control in analytical paper electrophoresis (method 1A). After separation, the solutions have been concentrated by dialysis against 20% dextran dissolved in veronal buffer solution. It is impossible to obtain perfectly pure α_1-globulin, and the separation between α_2-globulin and β-globulin is also incomplete. The adsorption which takes place between the paper and the lipoproteins (Kunkel and Slater 1952) also produces some inaccura-

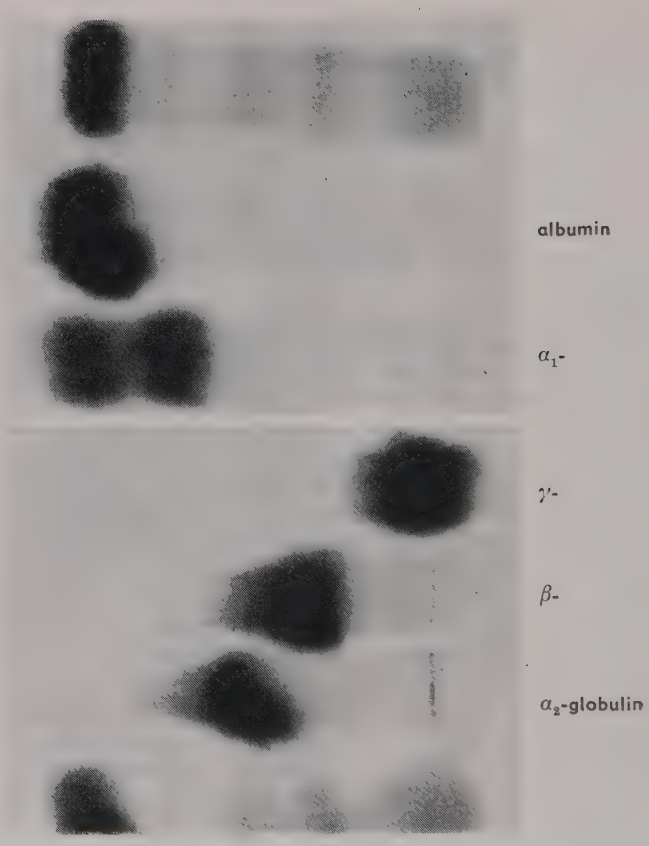

Fig. 12. Serum protein fractions isolated by preparatory electrophoresis (method 8 A) and run on paper along with the original serum (method 1 A).

cies in the determination of the lipoproteins in the fractions obtained by this method. The following procedure has been adopted in order to obtain control figures for the pH and ionic strength of the protein solutions: At the edges of the paper strips adjoining the protein fractions, samples of (non-protein) buffer were taken, and their pH and electric conductivity were estimated. The pH figures showed a decrease of about 0.16 units as compared with the original buffer; a little less at the side of the cathode adjacent to the γ-globulin. The conductivity showed an increase of about 10% over that of the original buffer. In consideration of the risk involved in delayed examination of the already prepared protein, mentioned above, no further dialysis was carried out after the isolation.

B. *Preparatory electrophoresis in starch (method according to Kunkel and Slater 1952, and Kunkel 1954)*

In this series of experiments the electrophoreses took place in a refrigeration chamber, and the serum was dialysed 24 hours beforehand against the veronal buffer (pH 9.1).

The starch (insoluble starch for chromatography, Grave) is first equiliberated with the buffer by the suction of 2–3 double volume buffer over the starch on a fritted-glass filter. The starch is then transferred, while still slightly moist, to a beaker, and buffer is then introduced, a small quantity at a time, the whole being continuously stirred. The amount of buffer added must be just sufficient to produce a smoothly-flowing liquid. A plastic cloth large enough to enwrap the cake of starch later, is laid on a glass slide of the dimensions given in 8A. Rulers are placed alongside the slide, and a thick filter paper folded at right angles is laid against each end. The starch colloid is then poured on to the plastic cloth and left to dry. It is most important that the table on which the preparation is performed should be perfectly horizontal, so that the starch will solidify to an even thickness. When the superfluous buffer (perceptible in the form of a liquid "mirror" in the centre) has been absorbed, the preparation will be ready for the application of the serum. A channel is formed with a spatula in a suitable position on the cathode side (near an imaginary line drawn between one-and two-thirds of the entire length of the cake of starch), and the serum is allowed to drop on to it. As a rule, about 5 ml of serum are separated. Filter paper can be laid on both sides of the channel in order to absorb the superfluous buffer. The channel is then closed with starch taken from the edge of the cake. An extra piece of narrow, slightly-moistened plastic cloth is now carefully put on top of the preparation so that no layer of air-bubbles can be formed between the top of the cake and the cloth, and finally the whole is enwrapped in the broad plastic cloth. Contact is established between the cake of starch and the buffer-containing vessels by means of damp linen cloths. The glass slide can usually be laid flat on the edges of the buffer vessels, so that the jelly is only two centimeters above the level of the buffer solution. A potential of 500 V (10 V/cm), giving a current of 60 mA is then applied. The electrophoresis runs for about 24 hours.

The cake of starch is divided into 1 cm pieces. The elution of the protein is carried out by packing each piece of starch in a fritted-glass filter into which buffer is then poured; after that the solution is sucked downwards and thus the elution becomes roughly quantitative. The protein concentration is then determined according to Method 11.

This method is an improvement over zone electrophoresis on paper as the higher degree of transparency in the stabilising medium renders the protein spots more clearly discernible when lit from below. In starch

preparation the haptoglobin in α_2-globulin is also easily seen as completely separated from the free hemoglobin in the β-globulin area, which is not the case in filter paper preparation. According to Kunkel, starch is the ideal stabilising medium for the zone electrophoresis of the lipoproteins.

Buffer: Veronal acetate buffer described on p. 20 was used.

Methods for estimation of proteins

Method 9

A modification of *protein combustion according to Kjeldahl*, described by Hiller, Plazin and van Slyke in 1948, has been used in order to standardise the protein methods 10, 11 and 12. The distillation has been performed in the apparatus described by Kirsten (1952); an ammonium sulphate standard comprising 1 mg nitrogen/ml has been included in each series of analyses. The protein content was obtained by multiplying the amount of nitrogen computed, by the factor 6.25. The error of the method was 0.079 g % protein (for a range of 0.4–11.3 g %; coefficient of variation 1.6%).

Method 10

The modification of the *biuret method* described by Levin and Brauer (1951) has been used for the estimation of the protein content of fractions obtained in preparative electrophoresis on filter paper (method 8A). The colour was recorded in a Beckman spectrophotometer, model B. Normal serum, preserved in a frozen state, has been used as a protein standard. The protein content of this serum has been estimated according to method 9 and the non-protein nitrogen by Rappaport's (1947) method. The variation in specific optical density which occurs between different kinds of serum protein fractions has not been taken into consideration in the estimation of the protein according to this method. Error of the method: 1.4 mg% (within a range of 13–137 mg%; coefficient of variation 2.0%).

Method 11

Estimation of the protein concentration in the serum protein fractions which have been obtained by preparatory electrophoresis in starch (method 8B) has been carried out by reading the optical density in *ultraviolet light* (284 mμ), against a buffer blank. By this method a rapid idea of the localisation of the various protein fractions on the diagram can be obtained. Method 12 was later used for more exact protein analysis. Readings were taken using a Beckman spectrophotometer, model DU.

Method 12

Total protein is estimated with *Folin's phenol reagent*, according to Lowry, Rosebrough and co-workers (1951), modified according to Egg-

TABLE 9. *Ratio optical density/protein concentration (acc. to Kjeldahl) in different serum protein fractions, determined with Folin's phenol reagent in the method modified by Lowry et al. All values are relative to albumin.*

A, B, C own values. C was obtained with a lyophilised preparation from AB Kabi, Stockholm. D, acc. to Brattsten (1955); E, acc. to Eggstein and Kreutz (1955).

	Albumin	α_2-glob.	β-glob.	γ-glob. (electrophoretically prepared)	γ-glob. (ethanol-precipitated)
A	1.0	1.06	1.40	1.60	
B	1.0	1.26	1.19	1.48	
C					1.53
D	1.0	1.1	1.3	1.6	
E					1.32

stein and Kreutz (1955). The method has been calibrated and controlled throughout with normal serum of known protein concentration. It has been used according to the authors' method; firstly, in a macro-modification in the protein concentration range of 40–270 mg% (error of the method 3.1 mg%, coefficient of variation 2.1%), and secondly in a micro-modification in a range of 0.6–9.8 mg% protein (error 0.09 mg%, coefficient of variation 2.8 %).

Estimation of specific optical density for the various protein fractions in serum.—Two experiments have been performed with preparatory electrophoresis in filter paper (A) and in starch (B). Phosphate buffer solution according to Sørensen, pH 8.0 and ionic strength 0.2 has been used. The protein fractions obtained have been concentrated according to method 14, and the protein concentration of these solutions estimated according to method 9. The protein solutions have then been suitably diluted, and the protein concentration estimated according to method 12 (the macro-modification). The quotient Q = (optical density)/(protein concentration) has been computed. The result of this series of experiments is given in Table 9, with Q indicated as relative to albumin (Q_{rel}). In the table some values from the literature are also cited. Hereafter the author applies the following values to Q_{rel}: 1.0, 1.1, 1.3 and 1.5 for, respectively, albumin, α_2-, β- and γ-globulin, being averages of the tabulated values.

METHOD 13. DETERMINATION OF SERUM LIPIDS

In a few experiments, the total lipid content of serum has been estimated by the method suggested by Swahn in 1953. It was calibrated with a serum, the lipid content of which was determined by a gravimetric technique (Brun 1939). This method has proved satisfactory as a first approximation, although open to criticism in some respects.

Method 14. Ultrafiltration

For the *concentration process* necessary to do some of the experiments in Chapters III and IV, ultrafiltration according to the method recommended by Aronsson (1957) has been used. In some of the fractions obtained afterwards, a small amount of precipitate, later removed by centrifugation, inevitably appears.

Method 15. Determination of pH

The *pH-estimations* have been carried out with a glass electrode in a pH-meter, Beckman model G, with an accuracy of 0.02 pH units. Two standard buffers have been used, potassium bipthalate buffer according to Michaelis, pH 4.0, and phosphate buffer according to Sørensen, pH 7.2.

Method 16. Micromethod for evaluation of protein (euglobulin), precipitated at low ionic strength, by turbidimetry

The method described below is a modification of "water dilution test at varying pH" (method 7, p. 23) adjusted to the low protein concentrations then available; thus the proteins are precipitated by a reduction in the ionic strength and a variation of the pH. It may be added that this method is unsuitable for the thymol and the zinc sulphate reactions, as the reagent alone produces a high degree of turbidity when analysed by sensitive recording instruments.

Reagents

All *water* used in these experiments was glass-redistilled. In the earlier experiments it was filtered through a fritted-glass filter (porosity G 4) in order to obtain a medium free from dust. However, this filtration was found to be superfluous; the water only required to be left to stand for about twenty-four hours before the experiment was begun, so that the sedimentation of the particles could take place.

The *hydrochloric acid* was in the majority of the experiments 0.02 *N*.

The *protein fractions* which had been prepared by electrophoresis according to 8A or B, were adjusted with veronal buffer to a protein concentration 100 times greater than the final figure. These protein solutions should be used as soon as possible after preparation. In order to remove microscopic impurities and possible traces of denatured protein, the solutions in question were centrifuged immediately before the experiment for 5–10 minutes at 10,000 r.p.m.

Apparatus

The investigation was carried out in a nephelometer designed by Stein and Doty (1946) and Hadow, Sheffer and Hyde (1947) in the modification described by Wallenius (1954). The turbidity was recorded in the form of light, scattered 90° against the incident beam of light. The very small amount of scattered light necessitates the use of a sensitive photo cell system (photomultiplicator cell I P21). The examination was carried out in monochromatic light (filter Zeiss C, 4358 Å). The degree of intensity of the dispersed light hereafter referred to as turbidity was directly recorded on a galvanometer, in relative scale units. In the course of the experiments the apparatus was frequently standardised by means of a polished plexi-glass block, the same size as the glass cuvette used in the experiments.

Procedure

The experiments were carried out at room temperature. The protein fractions selected for investigation were diluted 100 times, i.e. reduced to an ionic strength of about 0.001. The protein concentration in this dilution, the "primary dilution," was usually between 1 and 2 mg per 100 ml, with pH between 8.2 and 8.3. The turbidity of the primary dilution (=initial turbidity) maintains a stable level, with a value only slightly greater than that of pure water. 5 ml of the primary dilution was poured into the photometer cuvette and—when the initial turbidity had been read—a small amount usually about 0.03–0.05 ml, of hydrochloric acid was added. The micture was stirred with a glass rod, and the turbidity read after 1 minute. This sample was reserved for the determination of pH. In every succeeding determination, a slightly larger quantity of hydrochloric acid (with an increase of 0.01 ml each time to the final figure of 0.15 ml) was added to a further 5 ml of the primary dilution. After every addition of hydrochloric acid the mixture was stirred, the turbidity recorded and pH estimated. When the data thus obtained, i.e. the *increase* of turbidity above the turbidity of the primary dilution and pH figures, are put into graphic form, they show a symmetrical curve with a maximum over a certain pH of minimum stability (see Fig. 13). In most cases however, the curve was not continued after it had passed the turning point, i.e. had reached the acid side of pH of maximum turbidity. It is usually necessary to perform between 5–10 tests in order to obtain this curve. The values of the pH of maximum turbidity given in the following investigation thus correspond to the highest turbidity value found in each experiment. In order to give an estimate of the accuracy in the determination of this pH it might be said that an addition of 0.01 ml acid means a shifting of pH in the cuvette by 0.2 units (above pH 6.0 up to 0.2(4), below pH 5.5 down to 0.1(5) pH units). A graphical method for finding out this pH by interpolation was subject to the same amount of uncertainty. As the quantity of

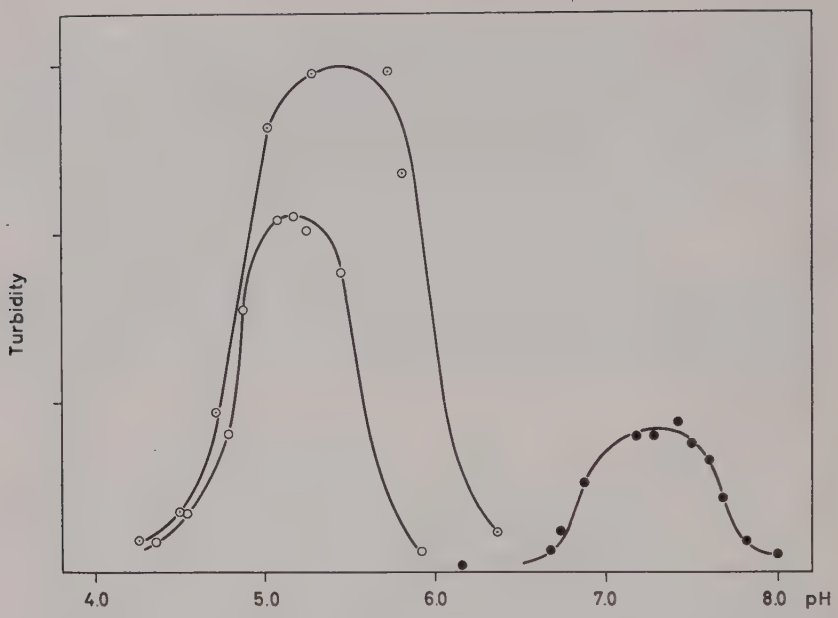

Fig. 13. Turbidity curves, obtained with serum protein fractions, isolated in zone electrophoresis, by precipitating at low ionic strength and varying pH. O, α_2-globulin; ⊙, β-globulin; ●, γ-globulin.

hydrochloric acid added to the dilutions was so small, the turbidity values were not corrected.

Various divergencies from the conditions described above were observed arising from the use of γ-globulin isolated from some cases of parenchymal liver injury, and also from that of the slow-moving subfraction of γ-globulin (γ_2), isolated from normal serum. In these cases, initial turbidity as defined above was not constant even at this alkaline pH, as an indication of the occurrence of some γ-globulin precipitation. Consequently, in later experiments, a small quantity of sodium hydroxide solution (2 ml $N/10$ NaOH per liter of water) was added to the water used for the dilutions. The pH of the primary dilution was thus adjusted to about 9.0, and the initial turbidity was now constant.

Error of the method

For turbidity estimations at pH of minimum stability the coefficient of variation reached the figure of 5.2 %. They were carried out as duplicate determinations, using identical primary dilution and identical quantities of acid.

Comments on the method: a discussion on turbidity

I. *A stable colloidal system (wherein turbidity is constant).*

When light passes through a colloidal solution it is diffused in all directions (Rayleigh scattering). In the case of very weak scatterings, e.g. in strongly diluted colloidal solutions it is preferable to measure that amount of diffused light, I_{90}, which travels at an angle of 90° towards the incoming light beam. Turbidity (τ) as used hitherto, in the ordinary lability reactions, denotes optical density and is recorded in the direction of incident light beam. Between τ and R_{90} a simple connection exists, when R_{90} is defined as $K\,(I_{90}/I_0)$, I_0 denoting the intensity of incident light and K a constant. In the investigation here described the expression turbidity is used to denote I_{90} and is expressed by means of arbitrary units.

If the colloidal particles are isotropic, spherically formed and, having regard to the wavelength of the light (λ), comparatively small (less than $\frac{1}{20}$ of λ), the following term (Doty and Edsall 1951, eq. 6 a) denotes R_{90}:

$$R_{90} = \frac{2\pi^2 n_0^2 \left(\frac{n - n_0}{c}\right)^2 cM}{A\lambda^4}, \qquad (1)$$

with n as the refractive index of the solution, n_0 the refractive index of the solvent, c the weight concentration of the colloid, $(n - n_0)/c$ the specific refractive increment for the colloid in the solution, M the molecular weight of the colloid, and A Avogadro's number.

Another important requisite to ensure the validity of the above equation is that the solution should be diluted to such an extent that no interaction between the colloidal particles can take place. Interaction is used here to denote any divergence from the random arrangement of the colloid particles (divergence from an ideal solution in the thermodynamic sense of the term). Should there be an attraction between the colloidal particles, the turbidity would rise above the theoretical norm, whereas repulsion would result in lower than theoretical turbidity.

II. *A colloidal system in process of precipitation*

In principle, the formula presented above is valid from moment to moment for a system in which aggregation is taking place. However, when the particles increase in size in proportion to the wavelength of the light, equation (1) will be affected so that λ will occur in a lower potency than 4 (cf. Oster 1948, Putzeys 1950). A growing particle will, therefore, exhibit a maximal scattering at about the stage in which its diameter is equivalent to λ (Caspersson 1933). In large particles another form of disturbance in light scattering also occurs, as the scattering varies greatly in strength when observed from differ-

ent angles. This asymmetry in the scattering causes the light to be spread more in a frontal direction than backwards or sideways.

Equation (1) shows how the turbidity, at every moment, is dependent on the molecular weight of the particles, or, in the case of a system undergoing aggregation, on their size. This dependence can be illustrated by a simplified version of Rayleigh's formula, as follows (cf. Oster 1947, eq. 1):

$$\tau = HNV^2, \tag{2}$$

where N represents the number of particles, V the volume of each particle and H a constant. Since NV is constant in an aggregating system the turbidity increase $(\tau_1-\tau_0)$ will be determined during a short initial period by the volume increase (V_1-V_0) according to the following equation:

$$\tau_1-\tau_0 = h(V_1-V_0), \tag{3}$$

h being a constant. This simplified formula is valid only for small particles. Should the particle volume even be comparable to λ, the turbidity increase would no longer be proportional to r^3, but to r, with r representing the radius of the increasing aggregate. With still bigger particles the light scattering will decrease again (Oster 1948).

Turbidity as a function of time during the precipitation of proteins

In the preceding work, in which the terms "lability" and "stability" were discussed, no attempt was made to give them more exact definitions. A clear and generally applicable interpretation has been provided by Overbeek (1952a), viz.: "The stability of the (hydrophobic) colloids is to be judged by their rate of coagulation (flocculation)." Measurements of turbidity were formerly much used in protein chemistry for studies in both colloid stability and the course of precipitation (Scatchard, Gibson, Woodruff and co-workers 1944, Heller and Klevens 1945, Oster 1947, Bier and Nord 1949, Edsall and Lever 1951, Foster and Rhees 1952, and Ressler and Jacobson 1956). The fundamental significance of aggregation speed for the determination of protein stability has been the guiding principle followed by the author in the formulation of his micromethod, and will be illustrated in the investigations described below.

The author's investigations

The protein fractions have been isolated according to 8A. They have been examined by means of turbidimetry (method 16) in order to ascertain the pH of minimum stability. Turbidity has then been observed at this pH for periods up to 1 hour. As illustrated by the curves in Fig. 14 a rapid initial turbidity increase takes place, followed sometimes by a slower rate of progress, sometimes by a constant level of turbidity and, in a few cases, by a slight decrease. A gradual increase of turbidity has

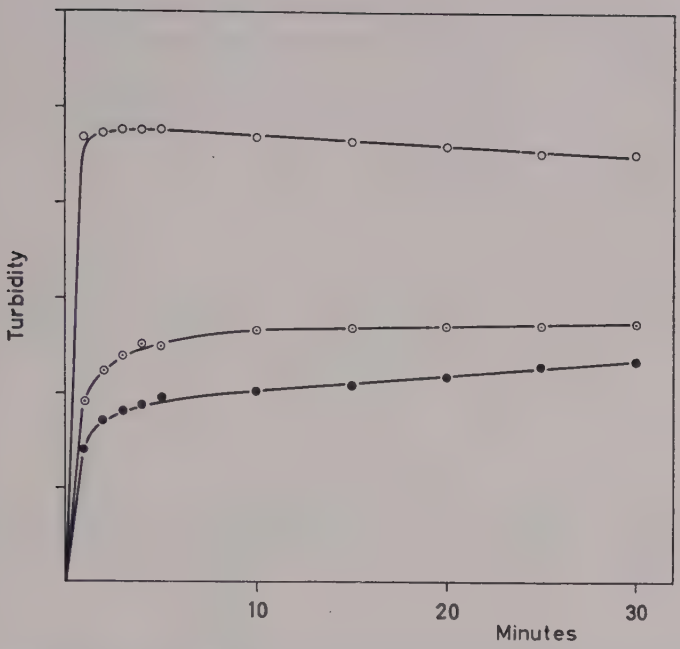

Fig. 14. The change in turbidity due to the change of time. Experiments performed at pH of minimum stability. O, α_2-globulin; ⊙, β-globulin; ●, γ-globulin.

usually been observed with the γ-globulin fractions examined in these investigations, lasting even longer than 30 minutes; whereas a rather stable level was often rapidly attained by the α_2- and β-globulins.

Discussion

In the section above, it was emphasised that the use of turbidimetry in the study of precipitation is subject to some disadvantages, especially when the recording of the dispersal of light takes place within 90° angle to incident beam. The variations of turbidity with increasing particle volume were also stressed. The aggregate increase (eq. 3) is only reflected accurately by the turbidity figure in the brief initial phases, before the aggregate has attained a certain dimension which is decisive in this respect. The level shown in some of the curves probably represents, not the terminal point of aggregation, but a retardation of it, and also serves to indicate that the "crucial" dimension has been attained. Changes in the optical qualities of the precipitate due to age or denaturation may also be contributary factors. The author has accordingly ascribed fundamental importance to the necessity of taking a reading at the earliest

possible moment. The period of 1 minute (60 seconds) has accordingly been selected for the method; at this moment the turbidity is still increasing, but slowly, and this mode of procedure gives satisfactory reproducibility in the duplicate experiments. Another alternative, of recording turbidity after 30 minutes, has never given, in the author's experience, good and reproducible precipitation curves.

Chapter II. The effect on the precipitation of serum protein fractions from changes in protein concentration, ionic strength and pH

1. *The importance of protein concentration*

During the preparation of the method for turbidimetry on a microscale, certain observations were made with regard to the importance of the protein concentration, which will be described below.

The author's investigations

The serum protein fractions were isolated according to method 8AI. Each fraction was examined in several different concentrations at an ionic strength of 0.001, and the turbidity at pH of minimum stability thus obtained was graphically contrasted with the protein concentration. As Fig. 15 shows, there is no linear connection between turbidity and the protein concentration in γ-globulin, whereas an approximately linear connection is usually obtained with the α_2- and β-globulins. The divergence from linearity with γ-globulin was such that increasing protein concentration resulted in an appreciably higher turbidity.

In order to discover whether the same conditions would prevail in the thymol and zinc sulphate reactions carried out with higher protein concentrations these reactions were subjected to investigation with varying total protein concentrations. Serum from a case of cirrhosis of the liver with positive reactions has been tested in various dilutions with saline; lability reactions have then been carried out on the dilutions. The various serum dilutions have been used as blanks in the photometer readings. There was some deviation from linearity in the direction described above, but in most cases it was only slightly significant. Within the limits of concentration subjected to examination (2.5–10 g %) an increase to 4 times the total protein concentration caused a 4.4 times increase of turbidity, i.e. about 10% more than would have been the case if there had been no deviation from linearity (cf. also Fig. 10, part I).

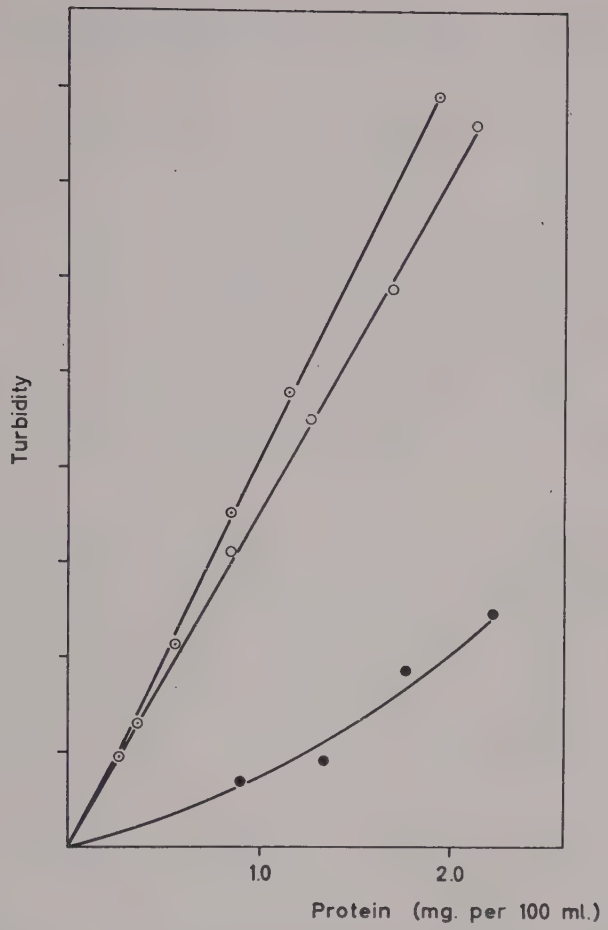

Fig. 15. Turbidity at pH of minimum stability as a function of protein concentration. O, α_2-globulin; ⊙, β-globulin; ●, γ-globulin.

Discussion

The phenomenon described above, interpreted as signifying that with an increased protein concentration a relatively greater increase will take place in the amount of precipitate obtained, has earlier been observed chiefly in the precipitation of serum proteins with neutral salts at high ionic strength. Consequently, when the albumin/globulin ratio is defined, this ratio will be found to decrease with higher total protein concentration, even if the reciprocal relations of the albumin and globulin in the solutions have not been altered (Chick and Martin 1913, Sørensen 1923,

Geill 1928, Yde 1937). Within the author's knowledge, no similar experiments with protein precipitation by reduction of ionic strength have been published.

The physico-chemical background of these relationships has been discussed by Sørensen in 1923, by Kunitz and Northrop in 1938, by Cohn and Edsall in 1948 and by Li in 1951. The fact that proteins are precipitated from a solution by alterations in the ionic strength (a decrease in the "salting-in" area, an increase in the "salting-out" area) indicates that with the altered ionic strength the solution is saturated as regards protein. When the ionic strength has been changed, an increased protein concentration in the original solution should produce a similar increase in the quantity of protein precipitated. But the fact that only a pure and well-defined protein substance can pass this test has been demonstrated. Should the protein selected for examination actually consist of several components of divergent solubility, increasing total protein content will produce a parabolic precipitation curve, as described here, if the ingoing proteins do not influence each other's solubility.

An exact linear correspondence between the precipitated protein and the total protein concentration cannot therefore be anticipated in experiments of this type with "crude" fractions of protein. It may be worthy of note that the greatest divergence from linearity was obtained with the γ-globulin which constituted the widest fraction on the electrophoretic separation. Succeeding experiments have invariably been carried out with identical protein concentration, whenever such a procedure was considered significant with regard to the presentation of the problem.

2 *a. The influence of changes in ionic strength on the turbidity, obtained by globulin precipitation at pH of minimum stability*

The solubility of globulins, and consequently also their stability in solution, is closely connected with the type and degree of concentration of other ions in the solution. This is partly due to the general ion effect exercised by the "neutral salts", which is a resultant of the electrolyte concentration and of the valencies of the ions in the solution. (Mellanby 1905). Increasing ionic strength causes an increase in the solubility of the serum proteins (the "salting-in" effect) up to a certain point, but beyond that point the solubility decreases again ("salting-out"). A more detailed treatment of the physico-chemical theory of the extent to which colloidal stability and solubility is determined by ionic strength will be found in the works of Cohn and Edsall (1948) and Overbeek (1952b). But certain metals (heavy metals, zinc, mercury, and alkaline earth metals) have a precipitating effect on some serum proteins, an effect much greater than the presumptive one obtained by computations of

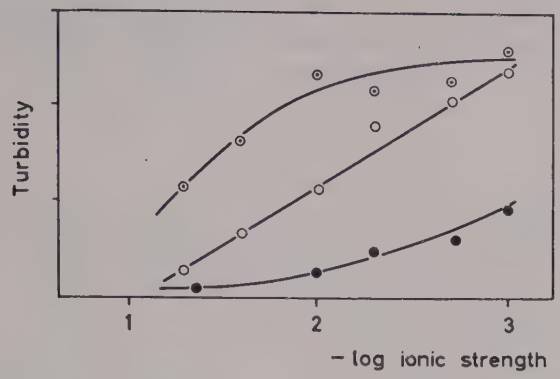

Fig. 16. Turbidity at pH of minimum stability as a function of ionic strength. O, α_2-globulin; ⊙, β-globulin; ●, γ-globulin.

ionic strength; it must therefore be regarded as a specific interaction between protein and ion (Cohn 1953). A study of this particular ionic effect, which is used in many flocculation reactions, is not, however, within the scope of the present work. Another aspect of the interaction between globulins and the electrolyte anions is given under section *2b*.

Flocculation reactions are usually carried out at an ionic strength, varying from 0.2 to 0.002 (cf. Maclagan's abstract 1948). The significance of the ionic strength for precipitation is extremely well illustrated in the water dilution test. If this test is carried out with constant protein concentration and at the pH of minimum stability, an uninterrupted increase of turbidity without perceptible maximum will be obtained when the ionic strength is decreased (Adner, unpublished experiments). In the following section some experiments will be described wherein isolated serum protein fractions were submitted to precipitation at pH of minimum stability and at different levels of ionic strength.

Author's investigations

Experiments have been carried out with serum protein fractions isolated according to method 8A. Turbidimetry (method 16) has been performed at different low ionic strengths, but with the same protein concentration 1 mg per 100 ml. The turbidity values obtained at pH of minimum stability are to be found in Fig. 16. On the graph, the data are better visualised by putting the turbidity values opposite to the negative logarithm of the ionic strength. To sum up, it may be said that within the area of investigation the turbidity of β-, and sometimes also of α_2-globulin shows a marked initial increase with diminishing ionic

strength, but later, in some cases, remains at a fairly stable level. The turbidity of γ-globulin, on the other hand, increases rather slowly with diminishing ionic strength, and does not attain a stable level.

Discussion

The fact that the most crudely dispersed (high-molecular) serum proteins are the first to be precipitated with decreasing ionic strength has often been stressed in the discussion of the chemical background of the flocculation reactions. This is also found to occur in the case of the high-molecular γ-globulins (Waldenström 1944). This phenomenon may, moreover, be analogous to the conditions prevailing in the related fields of macromolecular chemistry, wherein precipitation studies have been used in order to determine the molecular weight of polymers (cf. Morey and Tambleyn 1945). The fact that this simplification of the problem is not permissible with regard to the serum proteins and their complicated structure must, however, be emphasised. The lipoproteins within the α_2- and β-globulin group bear the character of "euglobulins" but this is certainly due to their high fat content rather than to their molecular weight.

If the investigations described above were applied to the water dilution test, and to other reactions resembling that test, the conclusion would be that with a decrease in ionic strength (within the "salting-in" zone) at first only the α_2- and β-globulins would be involved. The lower the ionic strength at which a test is carried out, the larger will be the proportion of the γ-globulins precipitated. In whole serum, however, somewhat divergent conditions prevail, due to protein-protein interaction, the dependence of which on ionic strength will be especially emphasised later.

2 *b. The connection between ionic strength and pH of maximum turbidity*

The general rule according to which the isoelectric point and, consequently, the pH of minimum stability undergo a shift towards acidity when the ionic strength is increased had been recognised since the era of Hardy's (1905) classical investigations (cf. Grönwall 1940). The data shown in Table 10 and taken from Table XV in the work of Alberty (1953) indicate the proportions which the shift in pH can attain in some serum proteins. A decrease in ionic strength from 0.10 to 0.01 will accordingly cause a shift of the isoelectric point of γ-globulin amounting to 0.8–0.9 pH units. An interaction between the protein molecule and anions in the solution has been said to give rise to this phenomenon.

A scrutiny of the experiments under *2a.* revealed that similar data could be derived in the author's system.

TABLE 10. *The shifting of pH of minimum stability with decreasing ionic strength.*

Compiled after Alberty, 1953.

	Ionic strength	Isoelectric point electrophoretically determined
Bovine serum albumin	0.10	4.71
	0.02	4.89
Human γ_1-globulin	0.10	5.8
	0.01	6.6
Human γ_2-globulin	0.10	7.3
	0.01	8.2

AUTHOR'S INVESTIGATIONS

The protein fractions have been isolated in veronal buffer with an ionic strength of 0.10, according to method 8A. The globulin fractions were submitted to precipitation as in the previous experiments; the protein concentration was kept constant throughout the experiment. A curve of precipitation was drawn and the pH of minimum stability ascertained for each ionic strength investigated. Some results of these experiments are reproduced in Table 11. The ionic strengths have not been adjusted for the addition of hydrochloric acid. These values reveal

TABLE 11. *The shifting of pH of minimum stability with decreasing ionic strength.*

Approximate ionic strength	pH of minimum stability			
	α_2-glob.	β-glob.	γ-glob.	
0.05	4.41	4.82	5.89	6.38
0.025	4.42	4.89		
0.010	4.59	4.89	5.97	6.50
0.005	4.75	4.92	6.81	6.48
0.002	4.68	5.08	6.58	6.71
0.001	4.69	5.04	6.95	7.35
0.0005		5.19		

quite clearly how the pH of minimum stability shifts in an alkaline direction when the ionic strength diminishes. A decrease in ionic strength from 0.05–0.001 causes a shift in this pH of γ-globulin of rather more than 0.8 units, in the material used by the author.

Discussion

Although the data cited above merely serve to confirm factual information which, from a physico-chemical point of view is already well-documented, the author has been convinced of the urgent necessity of recapitulating them for the following reason. In the first instance, many flocculation reactions take place near the point of neutrality, so that these tests must inevitably be especially predisposed to precipitate γ-globulin, because of the position of the pH of minimum stability in this globulin (see below).

But the appreciable shift of this pH towards neutrality or slight alkalinity with progressive dilution of the solvent will evidently contribute to increased precipitation of γ-globulin in tests performed at low ionic strength. The thymol and zinc sulphate tests take place at about the same pH in the neighbourhood of the neutral point, but at different ionic strengths. As may be gathered from the information provided above, they can quite justifiably be supposed to precipitate different parts of γ-globulin.

3. *The pH of minimum stability (maximum turbidity) of the different serum globulins at fixed ionic strength and protein concentration*

The greater part of the modern investigations on the isoelectric point of the serum proteins have been carried out by means of electrophoresis with an ionic strength of 0.10–0.20. Stenhagen (1938) accordingly gives the pH of 4.5, 4.8, 5.2, and 6.4 for albumin, α-, β- and γ-globulin respectively. A survey of the isoelectric points of the different plasma proteins fractionated according to the method employed by Cohn will be found in Table 12, which has been compiled in accordance with Cohn *et al.* (1950) and Hughes (1954).

The isoelectric points are shown scattered over a wide pH range. This applies particularly to the γ-globulins. Cann, Brown and Kirkwood (1949) have separated bovine γ-globulin into 8 fractions with isoelectric points varying from pH 5.7–7.3, and utilising the same technique, divided antipertussis γ-globulin into 4 fractions with isoelectric points ranging from 7.0 to 7.9 (1950).

Alberty, Anderson and Williams (1948) have found the distribution of the isoelectric point of γ_2-globulin to be from pH 6.3 to pH 8.4, with an

TABLE 12. *The isoelectric point of some serum protein fractions.*
Compiled after Cohn *et al.* 1950 and Hughes 1954.

Component	Approximative isoelectric point
Albumin	4.7–5.2
α_1-globulins	
α_1-mucoprotein	3
α_1-lipoprotein	5.2
coeruloplasmin	4.4
α_2-globulin	
α_2-glyco- and mucoproteins	4.9
β-globulin	
β_1-lipoprotein	5.4
lipid-poor β_1-euglobulin	5.5
transferrin	5.8
γ-globulin	
γ_1-globulin	ca. 6
γ_2-globulin	6.5–8

average pH of 7.3. Cohn, Deutsch and Wetter (1950) give the average isoelectric point for γ_1-globulin as pH 5.7, and that of γ_2-globulin as pH 7.5 with a broad distribution (from pH 5.0 to pH 8.4).

In the following section the aim has been to define the pH of minimum stability at low ionic strength for serum globulin fractions, isolated by preparatory electrophoresis.

AUTHOR'S INVESTIGATIONS

The author, as earlier related in the chapter on methods, has isolated serum protein fractions by means of two different processes; either eluting the protein fractions in bulk (method 8AI), or dividing the electrophoretic preparation into several cross-segments (method 8AII). Each fraction or segment was then subjected to precipitation, as described in method 16, and the pH of maximum turbidity was determined, with due respect to the uncertainty of the method. In all these experiments the protein concentration was 1 mg per 100 ml, and the ionic strength about 0.001. The data tabulated are obtained in experiments with normal sera (blood donors). The paper electrophoresis strips reproduced (corresponding to cases number 3, 9, 10, and 11) were run in veronal buffer, or in Fig. 18 A, in the buffer described on p. 20.

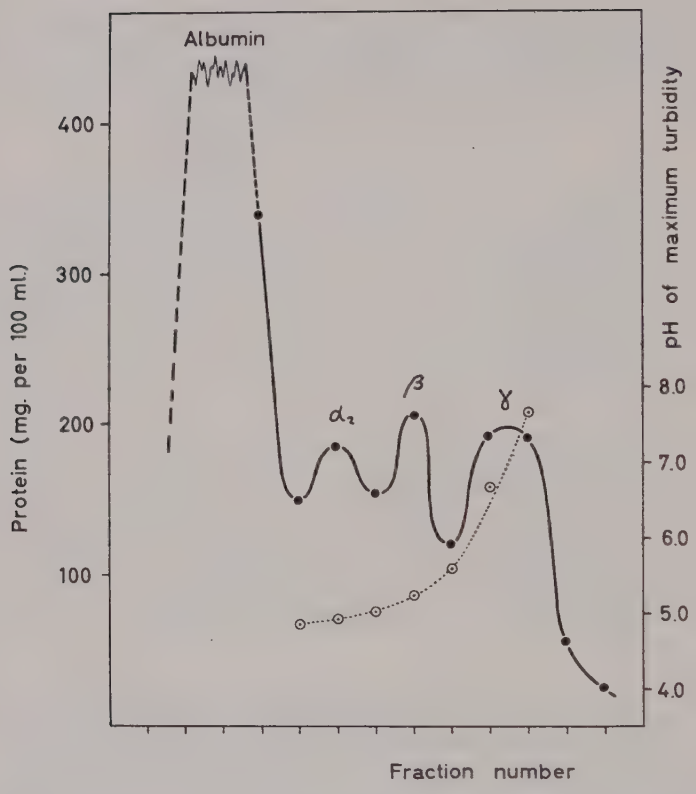

Fig. 17. Graph showing the pH of minimum stability (⊙) of different serum globulins (●) at ionic strength 0.001. The globulins are arranged according to electrophoretic mobility.

As is illustrated in Fig. 17, the precipitation optimum of the γ-globulin will accordingly occur over a wide pH interval, whereas that of the other globulins varies within a comparatively narrow pH range. In Table 13 a summary of all investigations carried out is presented. In the upper section of the table, with the fractions taken out "in bulk", the values have been summarised as the mean. The lower section of the table, which is based on curves of the type recently shown in Fig. 17, shows the experimentally detected values for pH of minimum stability of peak fractions in each of the globulins.

As mentioned in the section on methods (p. 52) the γ-globulins isolated from three cases of liver parenchyma diseases (cases nos. 6, 7 and 8) according to the method 8AI, showed a pH of minimum stability with a slight alkaline deviation from normal (7.5, 7.6 and 7.7). The significance of this deviation will be further discussed below. In connection with this

TABLE 13. *The pH of minimum stability of serum globulin fractions, precipitated at ionic strength 0.001 in a concentration of 1 mg per 100 ml.*

Exp. No.	Method	pH of minimum stability		
		α_2-glob.	β-glob.	γ-glob.
1–13	8 AI			
mean		4.9	5.2	7.0
range		4.6–5.0	5.1–5.3	6.6–7.3
14[a]	8 AII	5.0 (5.1–4.6)	5.5 (5.9–5.1)	7.0 (5.9)
15		4.9 (5.0–)	5.2 (6.0–5.0)	7.3 (6.0)
16		5.0 (5.1–4.8)	5.5 (6.1–5.1)	7.0 (6.1)
17		4.9 (5.0–4.6)	5.2 (6.0–5.0)	7.3 (6.0)
18		4.9 (5.0–4.8)	5.2 (5.6–5.0)	7.0 (5.6)
19	8 B	5.0 (5.1–4.9)	5.2 (6.0–5.1)	6.9 (6.0)

[a] Values within brackets represent the limits to adjacent fractions, obtained by analysing the fraction of the "valleys" between the globulins.

finding the author has endeavoured, by means of paper electrophoresis, to ascertain whether it might be possible to observe a dominance of γ_2-globulin in the γ-globulin from cases of liver parenchyma diseases. Occasional variations have been introduced into the method: in Fig. 18 some of the most typical curves are shown. In certain cases most remarkable changes can be observed, such as a slowing in mobility in the pathological, as compared with the normal γ-globulin and/or a slight splitting of γ-globulin to form a separate γ_2-fraction.

In another experiment preparatory electrophoresis in starch was performed on two sera, one being normal and the other hepatitic (case no. 12) under conditions as nearly identical as possible (method 8B). The γ_2-globulin was extracted in four consecutive fractions from the slow part of the γ-globulin; the γ_1-globulin included the top fraction and the three successive fractions of the more rapid part of the γ-globulin. The pH of minimum stability for the fractions isolated from normal serum was ascertained to 6.9 (γ_1) and 7.6 (γ_2), whereas for the fractions taken from hepatitic serum the figures were, respectively, 7.2 and 7.9.

DISCUSSION

The data of pH of minimum stability for normal serum protein fractions presented in the above account are evidently factors of fundamental importance for the results of the lability reactions, and especially for the influence exerted by the γ-globulin on these reactions. The difference which has been observed between the results of the thymol and the

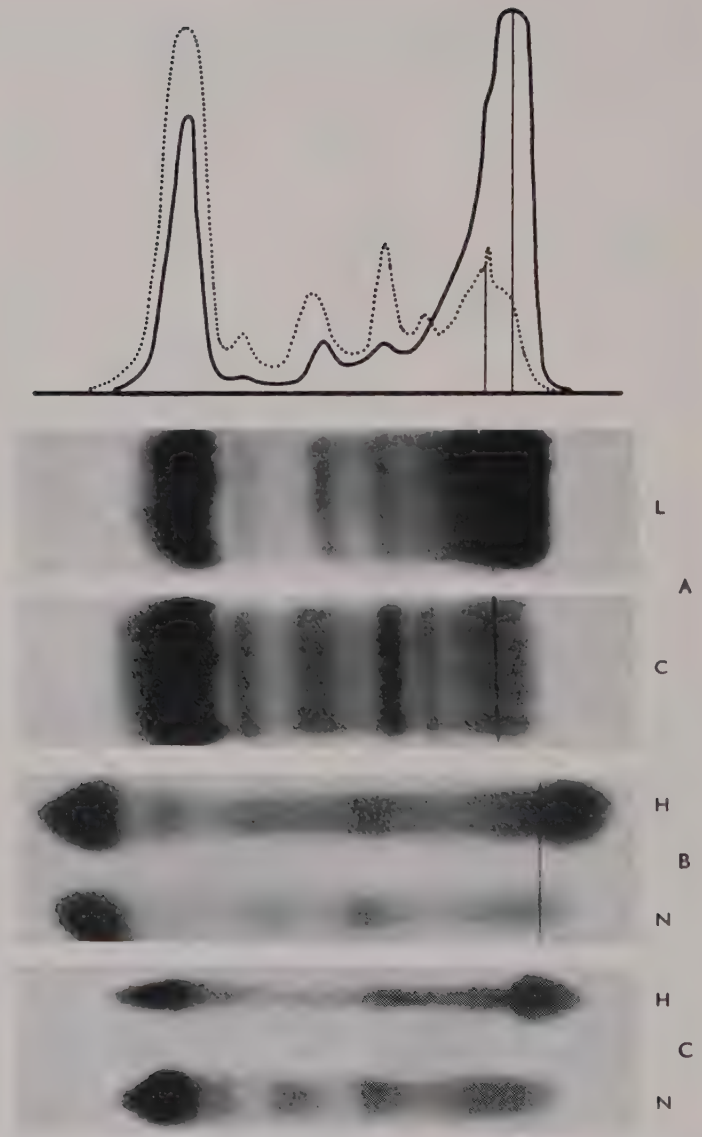

Fig. 18. The behaviour of γ_2-globulin in some cases of disease of the liver parenchyma, illustrated by paper electrophoresis. (A) Sera from a case of cholecystitis, C, and a case of liver cirrhosis, L. The vertical lines correspond to the peak of the main fraction of γ-globulin. (B) Sera from a case of chronic hepatitis, H, and a normal case, N, applied as a short streak on the same paper strip. (C) Sera from a case of acute hepatitis, H, and a normal case, N, applied as a spot on the same strip. (A) method 1B. (B) and (C), method 1A. The line on the strips denotes the starting-point.

zinc sulphate reactions may possibly be due to the fact that these reactions, which take place in buffers with slightly dissimilar pH, accordingly provide optimal conditions for the precipitation of *different* sections of the γ-globulin (cf. Franklin and Kunkel 1956). The entire problem is, however, considerably complicated by the co-precipitation which takes place between γ-globulin and other serum proteins (see chapter on interaction), as a consequence of which the pH values of minimum stability cited above, are not applicable to precipitation in whole serum.

Several observations presented in the survey of the literature given in the introduction to part I indicated that, in liver parenchyma diseases, the γ-globulin was more "labile" than under normal conditions of health. The recently mentioned data would not only be consistent with these observations but also provide an explanation for them. The only objection to this theory is the difficulty of excluding the possibility that the procedure of isolating the γ-globulin may have had some effect on the result of the experiment. If, for instance, the γ-globulin is taken "asymmetrically" from the zone electrophoresis, so that there is a preponderance of γ_2-globulin, the result of the experiment will, of course, be affected by this to a certain extent. Some objections can also be made to the analytical electrophoretic investigations. Even a difference in concentration between γ-globulin from different sera can provide electrophoretic pictures resembling those presented above, as a result of divergent diffusion of proteins in all directions. In this case, however, the peak of the γ-globulin should invariably be found in the same position. It should be emphasised that even the slightest deviations in mobility within γ-globulin are worthy of note, since this fraction covers so wide a pH-range in relation to the isoelectric point (see Fig. 17). In no way can the final solution of this problem be said to have been attained as yet; indeed, it cannot be solved without a method of more complete fractionation of γ-globulin.

Franklin and Kunkel (1956) present similar observations. In paper electrophoresis of sera from portal cirrhosis, Satoskar, Lewis *et al.* (1954) perceived a demarcation between γ_1- and γ_2-globulin. The question of γ_1- and γ_2-globulin will be clarified from another aspect in the chapter dealing with protein-protein interaction.

Chapter III. The influence of lipoproteins on turbidity, obtained in precipitation of serum proteins at low ionic strength

In the preceding chapters the term turbidity has been used as equivalent in meaning to "the quantity of precipitated protein". This method of naming is customary, not only in connection with the lability reactions,

but also in a number of turbidimetric micro-methods for the determination of serum proteins and their fractions (Salt 1950, Parfentjev and Johnson 1952, etc.). However, the value of this procedure is open to doubt.

The term turbidity was discussed from a physico-chemical point of view on p. 53. An attempt to standardise the applied turbidimetry with a more exact quantitative protein determination method will be described below. The object of the investigation was thus to study the correspondence between turbidity values and quantity of precipitated protein and, also, to find out the relative ease of precipitation (euglobulin content) of each serum protein fraction.

Author's investigation:

Method

1. The protein fractions, obtained from normal nonlipemic sera and from one case of hepatitis (case 12) were displayed by means of electrophoresis in starch (method 8B). The various fractions were obtained by "pooling" the protein, which had been recovered from the small segments of the electrophoresis, their localisation within the electrophoretic diagram being computed according to the curve obtained by ultraviolet photometry (method 11). The protein analysis was performed according to method 12 (the macro-modification), the specific optical density of the various serum protein fractions with the factors presented in the above-mentioned chapter, being taken into account. The fractions were then adjusted to 200 mg per 100 ml, and turbidimetry carried out according to the earlier descriptions, with a final concentration of 2 mg per 100 ml. The turbidity at pH of minimum stability was ascertained. The sample at this pH was retained, and the following standard procedure was utilised for the determination of the protein content of the precipitate.

The sample was left standing at room temperature for 30 minutes. Centrifugation was then carried out at 10,000 r.p.m. for 5 minutes. The supernatant was then decanted, and a colorimetric definition of the protein content of both supernatant and the "primary dilution" according to method 12 (the micro-modification) was performed; the protein content of the sediment being finally computed from the difference between the two analyses. These values were then translated into μg protein, taking into account the above named factors for specific optical density. The procedure has been subjected to control, with regard to the lapse of time between the addition of the acid to the "primary dilution" and the initiation of the centrifugation; it was thus discovered that an incubation period of over 30 minutes did not result in a higher yield of precipitate. Nor did an increase in time of centrifuging cause any increase in the quantity of precipitate obtained. Control of the turbidity also showed that after only one minute of centrifuging the turbidity

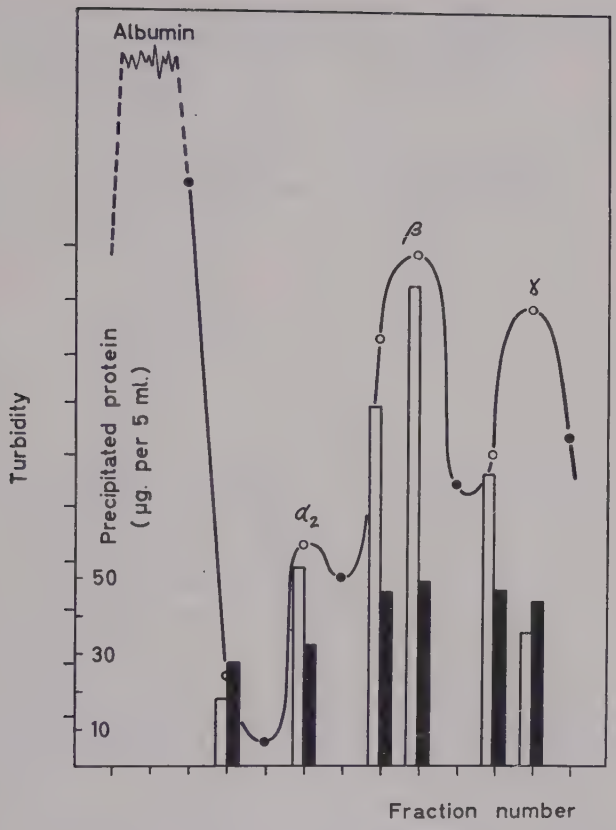

Fig. 19. Diagram showing the relationship between turbidity and precipitated protein of different globulins at pH of minimum stability. The globulins are arranged according to electrophoretic mobility. □, turbidity, ■, microg. precipitated protein per 5 ml solution. ○, fractions investigated by turbidimetry; ●, fractions not investigated.

had decreased to the same degree as that found in the "primary dilution". In the procedure described above, the error is 3.8 % (computed from a number of centrifugal tests performed on a single sample with precipitating protein).

2. Under this heading experiments will be described in which sera, obtained from a few cases with varying serum lipid content (cases 14, 15 and 16), including two blood donors, were subjected to preparatory electrophoresis in paper (method 8AII) and the isolated protein fractions further investigated. Total lipid content of serum was estimated with method 13 and total protein content with method 2.

TABLE 14. *The relationship between turbidity and quantity precipitated protein at pH of minimum stability.*

Values are average (extreme values in each series within brackets).

Fraction	Relative turbidity/μg precipitated protein per 5 ml solution	Protein precipitated as "euglobulin" in % of total protein present (ionic strength 0.001)	No. of analyses
α_2-globulin	2.4 (2.0–3.1)	39 (29–45)	3
β-globulin	4.4 (3.2–6.2)	43 (38–49)	7
γ_1-globulin	2.3 (1.5–3.2)	45 (31–53)	7
γ_2-globulin	1.3 (0.8–1.6)	32 (18–48)	5

RESULTS

1. If the fractions are classified in groups according to electrophoretic mobility, and the quotient of turbidity/quantity of precipitated protein is computed, the values recorded in Table 14 will be obtained. In the present examination, the hepatitis case did not differ in any respects from the other material except that it contained the highest recorded quotient in the β-globulin group. The dispersion of the values within each group is probably mainly due to the fact that it is extremely difficult to obtain the fractions from the electrophoresis with adequate reproducibility from time to time. For the estimation of the values it is important to remember that the boundary between γ_1- and γ_2-globulin is arbitrarily chosen. It is, however, obvious that the highest quotient is found in the precipitate obtained from the β-globulin[1] and that this globulin when compared with the γ_2-globulin shows an average figure 3.3 times as great as the latter globulin (with a maximum value of 7.6 times that of γ_2-globulin). The remarkable variation in the quotients from one fraction to another is also illustrated by the experiment reproduced in Fig. 19.

In order to show the proportion of the proteins which is precipitated as "euglobulin" according to the stipulations for this investigations (ionic strength 0.001 and pH of minimum stability), the precipitate has been calculated as a percentage of the original protein concentration. These figures are also given in Table 14. Particular stress should be laid on the greater yield of precipitate in the γ_1-globulin group as compared to the γ_2-globulin.

2. Some further investigations yield evidence of a certain connection between turbidity at pH of minimum stability of β-globulin and the

[1] This statement is made with reference to experiments carried out with fractions prepared from electrophoresis in starch. The earlier experiments with fractions obtained from paper appeared to indicate, that the fraction with the highest turbidity at pH of minimum stability was found midway between the α_2- and the β-globulin.

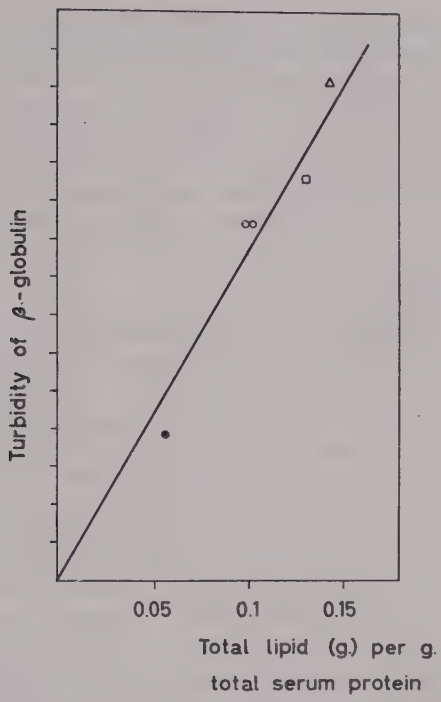

Fig. 20. The relationship between the turbidity of β-globulin at low ionic strength and optimal pH, protein concentration being 1 mg per 100 ml, and the total lipid content of serum (g/g total protein). •, acute hepatitis; □, coronary thrombosis; △, nephrotic syndrome; O, normal case.

lipid content of whole serum. The protein fractions were submitted to precipitation at low ionic strength and the highest turbidity value found (coinciding approximately with the β-globulin fraction) was compared with the lipid content per g protein in whole serum (cf. Fig. 20). Because of the small number of cases investigated these results should be considered as preliminary.

Here we may add that the slight opalescence seen in the fresh electrophoretically prepared fractions, mentioned in the chapter on methods, was approximately parallel to the turbidity later obtained in the precipitation experiments. This opalescence has also been interpreted as mainly due to the presence of lipoproteins (*vide infra*).

Discussion

The higher yield of euglobulin in the γ_1-globulin group as compared to the γ_2-group has been mentioned by Cohn, Deutsch and Wetter (1950).

They established by means of isoelectric dialysis the fact that (ethanol fractionated) γ_1-globulin contained 50–70% protein, insoluble in water, whereas only about 10% of the γ_2-globulin was insoluble in water. This circumstance may perhaps be ascribed to the content of globulins with a high molecular weight found within the γ_1-globulin group (Brattsten 1955, Wallenius, Trautman *et al.* 1956).[1]

The high specific turbidity found in the precipitate obtained from isolated β-globulin was mentioned above. The explanation of the findings presented here is based on the assumption that this precipitate is mainly composed of β-lipoprotein. According to Oncley, Gurd and Melin (1950) the lipid content of that lipoprotein may amount to more than 70%. In equation (1), on page 53, it was shown that turbidity is a function of the square number of the specific refractive increment of the colloid. Armstrong, Budka and coworkers (1947) have determined the refractive index for a number of proteins (sodium chloride solution, ionic strength 0.3, wavelength 5780 Å) and have then computed the specific refractive increment with reference to g nitrogen/litre. They found values varying from 1.17×10^{-3} for proteins poor in lipids and carbohydrates to 2.03×10^{-3} for crude β_1-lipoprotein (4.05×10^{-3} for purified lipoprotein). These values are not, of course, completely applicable to the author's system, but are at present the only ones available. If they are applied to eq. (1), p. 53, it will be seen that the turbidity (computed per g precipitated protein) can be 3–12 times greater for lipoproteins than for lipid-poor proteins. The values found in this investigation, which imply a more than threefold increase of turbidity per unit protein in the precipitate from β-globulin compared with the turbidity in the precipitate from γ_2-globulin, which is deficient in fats and carbohydrates, are thus fairly well explained. It is, of course, never claimed that the facts discussed here are the only relevant ones in this connection. The object has only been to point out the necessity for making allowance for the high lipid content of the precipitate.

The serum protein (euglobulin) obtained from whole serum at low ionic strength is mostly of β-globulin origin (Gurd 1949). The discrepancy between turbidity and precipitated protein described above will therefore be an important factor in the water dilution test and, consequently, in other lability reactions performed at low ionic strength, where the precipitate is evaluated by means of turbidimetry. By the use of these methods the variation in the lipid content of the β-globulin will be recorded with great sensitivity. In this connection the fact that Maclagan (1944) emphasised the importance of the lipid content in the thymol precipitate, and that Kunkel and Hoagland (1947) stressed the close connection between the lipid content of the precipitate and that of the

[1] In a high-molecular γ-globulin isolated by the author from a case of macroglobulinemia (case 13), 91% of the protein was precipitated in the conditions indicated here.

serum, should again be noted. The difficulties encountered in correlating lability reactions with the quantitative data from paper electrophoresis on serum can now be considered in relation to the circumstances described above, more especially as the β-globulin often shows a significantly high fat content in diseases of the liver. (See the survey of the literature appended to part I.) The high value of the quotient turbidity/protein content of the precipitate from β-globulin in one case of hepatitis corroborates the validity of this conclusion.

Chapter IV. The protein-protein interactions and their importance in the flocculation reactions

One of the characteristic qualities of the serum proteins is their capacity for coalescing more or less permanently with innumerable other substances. The term "interaction" (hereafter synonymous with "positive interaction", cf. p. 53) is used to denote this tendency. The type of interaction which will be specially considered here is the non-specific type which takes place between serum proteins and other highly-charged macromolecular substances, and which is mainly due to the electrostatic charge in the proteins. The inference thereby implied is that the proteins, when on the alkaline side of their isoelectric point, interact as negatively-charged particles, with positively-charged ions; whereas on the acid side they interact, as positively-charged particles, with negatively-charged ions. It is, however, necessary to emphasise the fact that due to the amphoteric properties of the proteins a protein molecule with a negative net charge may still contain a residuum of positive valences which suffices to bind a negative ion, i.e. an interaction to negatively-charged ions can even take place on the alkaline side of the isoelectric point of the protein substance (Stenhagen and Teorell 1939, Chargaff, Ziff and Moore 1941, Goldwasser and Putnam 1950, Haurowitz, Schwerin and Tunç 1946, Lauffer 1947, Turner and Boyer 1952, Blasius and Seitz 1952, Noguchi 1956, Jameson and Barnet 1957).

Earlier observations of this character have been made in connection with investigations of precipitation, solubility, or thermal denaturation. Using all the resources of physical chemistry, it has, however, been possible to study the phenomenon in its entirety. The existence of soluble complexes has, for instance, been proved by means of electrophoresis and ultracentrifugation, and other methods such as light scattering, have revealed the transient, reversible form of interaction, which can only be interpreted as "increased probability of approach between the colloids" (Doty and Edsall 1951, see p. 53). There is an enormous body of literature covering this field; for surveys, see Abramson, Moyer and Green 1942, Cohn 1953, and Klotz 1954.

Thus interaction between proteins and nucleic acids has been described by Kossel (1894), Michaelis and Davidsohn (1912), Hammarsten and Hammarsten (1928), Seibert and Watson (1941), Björnesjö and Teorell (1945), Goldwasser and Putnam (1950), Greenstein and Hoyer (1950), Singer, Timasheff and Kirkwood (1952) and by Hamer (1954), and interaction between proteins and nucleoproteins (tobacco mosaic virus) by Lauffer (1947). The combinations of proteins and macromolecular polysaccharides with a strong negative charge are an interesting group, on account of the important biological functions fulfilled by the latter. The group includes heparin (Chargaff, Ziff and Moore 1941, Blasius and Seitz 1952, Clarke and Monkhouse 1953, and Hamer 1954), chondroitin sulphuric acid (Mörner 1889, Meyer, Palmer and Smith 1937, Badin and Schubert 1955) and hyaluronic acid (Hamer 1954). A more heterogeneous group is formed by proteins combined with synthetic polyelectrolytes such as lysine polypeptide (Rice, Stahmann and Alberty 1954), polyglutaminic acid and dextran sulphate (Hamer 1954), polymetachrylic acid and polyvinylamide (Morawetz and Hughes 1952) and certain cellulose derivates (Noguchi 1956). Most interesting in this connection, however, is the protein-protein interaction (Kossel 1894, Bang 1899, af Ugglas 1914, McFarlane 1935, Haurowitz and Marx 1936, Kekwick and Pedersen 1936, Longsworth, Keith, *et al.* 1940, Grönwall 1940, Steiner 1953, Ross 1954, Jameson and Barnet 1957, and several others), more especially the works with the interaction between the various serum proteins (Cohn 1922 and 1953, Lontie and Morrison 1947, Oncley, Ellenbogen, Gitlin and Gurd 1952). The most exhaustive work on this subject is, perhaps, that of Bungenberg de Jong (summarised in 1949). He has utilised most of the systems described herein, and has also given special attention to the combinations of proteins and phosphatides, particularly lecithin (1931).

The conclusions reached by these authors with regard to interaction may be approximately summarised by the following points:

(1) The interaction is to a considerable extent determined by pH.

(2) An increase in ionic strength inhibits the interaction, a decrease promotes it.

(3) The interaction is stimulated by a high protein concentration.

(4) The proportions of the interacting proteins in the precipitate are dependent on the pH. If the pH is allowed to remain constant, the addition of a surplus of either component to the supernatant can result in the redissolution of the precipitate.

(5) Stress has also been laid on the fact that an interaction cannot always be found (Tiselius 1930, Kleczkowski 1946, and Cohn 1953), at least not as a precipitate, in the position in which according to the conditions postulated above, it should take place, i.e. in the pH zone between the isoelectric points for the interacting proteins. The occurrence of soluble "complexes" in this zone will be further discussed under E.

On the other hand, soluble "complexes" have been observed on several occasions in the pH zone outside the isoelectric points (cited above).

In the experiments to be related under sections A–F γ-globulin has been studied in its interaction with other isolated serum protein fractions at low ionic strength and with the turbidimetric technique previously applied to precipitation studies.

Author's investigations

Methods

The serum protein fractions obtained from sera of blood donors have been prepared by zone electrophoresis on paper or starch (method 8.). When the protein content was low they were concentrated according to method 14. In some experiments fractions (albumin and γ-globulin [AB Kabi, Stockholm]), prepared according to Cohn by precipitation at low temperature with ethanol, were used. They were obtained in a lyophilised state without preservatives. The mucoprotein was prepared from human plasma by the method described by Weimer, Mehl and Winzler (1950). Because of the small recovery it was, however, not reprecipitated with ammonium sulphate, but immediately after preparation dialysed and lyophilised. On paper electrophoresis the main fraction had the mobility of a fast-moving α_2-globulin. A faint band was also seen with β-globulin mobility. An analysis gave a content of 12% hexose (determined with the anthrone method as mannose-galactose according to Björnesjö 1955a), 9% hexosamine (in Elson and Morgan's method modified by Blix 1948), and 10.3% nitrogen.

The isolated fractions were investigated by turbidimetry (method 16). After that the fractions were mixed, the mixtures diluted with water and analysed turbidimetrically. The protein concentrations were so adjusted (except in the experiment described under C) that the final concentrations were the same for each fraction, isolated or in mixture. In some later experiments the study was also made more complete by an analysis of the precipitated protein using the method previously described (p. 68). Since the proportions of the different fractions in the precipitate were not known all values related to the precipitate were calculated with the factor attributed to γ-globulin (p. 49).

Results

A. *The protein inhibitors of the precipitation of γ-globulin*

1. In the experiment to be described all fractions were isolated by electrophoresis and they were all investigated at a single pH, 7.5. γ-globulin was studied alone and mixed with albumin, α_2- or β-globulin. At this pH, only γ-globulin gives a readable turbidity (cf. Fig. 13). The

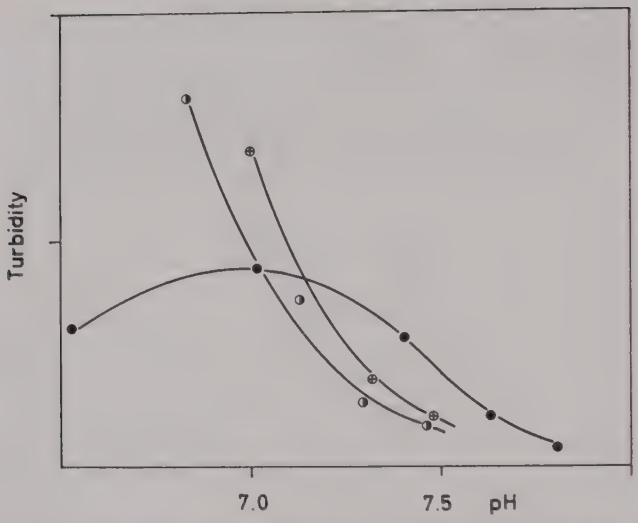

Fig. 21. Graph showing the interaction between β-globulin and γ-globulin in the region near neutrality. ⊗, β-globulin (0.2 mg per 100 ml) + γ-globulin; ◑, β-globulin (0.5 mg per 100 ml) + γ-globulin; ●, γ-globulin alone. Data of experiment A (2).

results are given in Table 15, A (1). The precipitated protein was spun down and the amount calculated. Albumin and α_2-globulin "inhibit" the precipitation of γ-globulin in this experiment and in the proportions used, but the inhibition displayed by β-globulin is only slight as determined by turbidity. If the precipitate is analysed, the inhibition is, however, of about the same magnitude for all fractions.

2. In another experiment, Fig. 21 and Table 15, A (2), the inhibition exerted by β-globulin is clearly demonstrated with turbidimetry in the same region of pH.

Comment.—If read at pH 7.5 the inhibition of precipitation of γ-globulin is thus effected by all proteins with an isoelectric point in the acid region. These proteins behave here like protective colloids. The difference between turbidity and precipitate demonstrated in the mixture of β- and γ-globulin may again be ascribed to the lipid content of the precipitate. With this technique, however, the important aspect of pH of minimum stability of the mixture of proteins is lost.

B. *The interaction as a function of pH*

This topic is illustrated by the experiment shown in Fig. 22 and Table 15, B, where an albumin (including α_1-globulin) fraction was mixed with a γ-globulin sample, both electrophoretically prepared. The albumin fraction gave practically no turbidity when examined alone. The

TABLE 15. *Interaction between γ-globulin and other serum proteins.*

Exp.	Fractions (all prepared by electrophoresis)	Protein concentration mg/100 ml	pH	Turbidity	Precipitate (μg protein/5 ml determined as γ-globulin)
A (1)	γ	3.7	7.5	94	35
	alb. + γ	1.8 + 3.7		35	17
	α_2 + γ	1.8 + 3.7		58	13
	β + γ	1.8 + 3.7		89	15
A (2)[a]	γ	0.9	7.3	16	
	β + γ	0.2 + 0.9		11	
	β + γ	0.5 + 0.9		7	
B	γ	0.8	7.0[b]	27	
	alb. + γ	5.5 + 0.8	6.0	84	

[a] Values interpolated from Fig. 21.
[b] Values for exp. B refer to pH of maximum turbidity.

γ-globulin gave a moderate turbidity with a pH of minimum stability at about 7.0. The mixture between the two fractions, however, gave a completely new precipitation curve, with a pH of minimum stability in the neighbourhood of 6.0.

The most conspicuous feature in this experiment is the high turbidity in the zone between the pH of minimum stability of each isolated fraction. In a zone, alkaline to the pH of minimum stability of γ-globulin, the precipitation of this fraction is inhibited by the presence of albumin. The same type of curve has been obtained in mixtures between α_2- or β-globulin and γ-globulin.

Comment.—The proteins of the mixture thus behave like a new protein with new qualities of solubility. When speaking of interaction, it must be emphasised that this effect is different at different pH. This, of course, may be explained by assuming the formation of insoluble "complexes" in one region of pH and soluble "complexes" in another region, the word complex here and later on used in the same wide meaning as interaction. The fact that the same protein at one pH may precipitate another protein, and yet at another pH, protect it from precipitation, is not always clearly pointed out in the literature. Its implications for the lability reactions cannot be overemphasised, especially with regard to the wide range of the pH's of minimum stability of the serum proteins (4.5 to about 8). The difficulty of correlating lability reactions to the electrophoretic serum protein fractions (part I) can partly, at least, be explained by the protein-protein interactions described above.

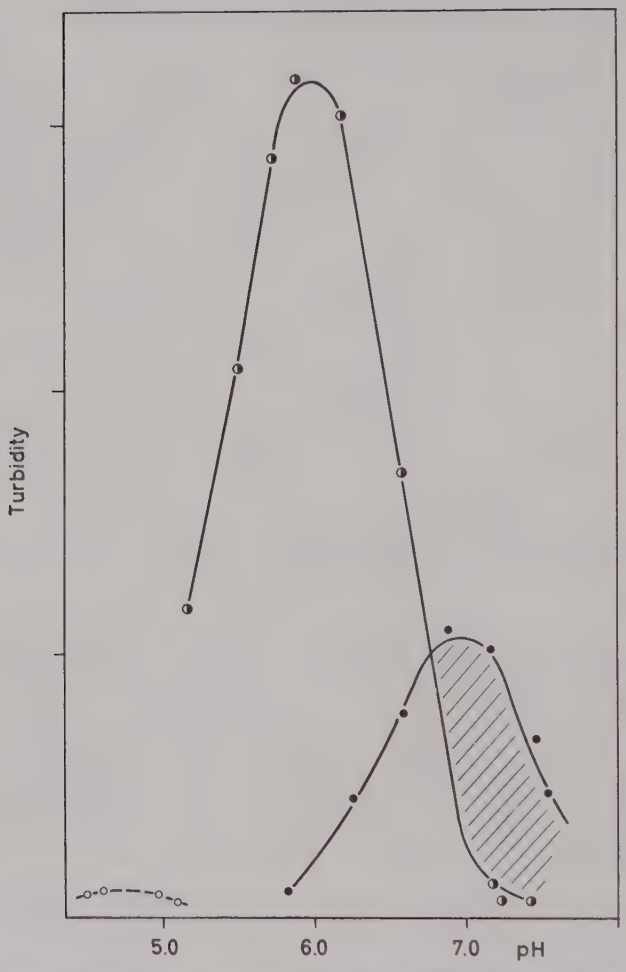

Fig. 22. Graph showing the interaction over a wide pH range between γ-globulin and an albumin prepared by electrophoresis. ○, albumin alone; ●, γ-globulin alone; ◑, albumin and γ-globulin in mixture. Striated area, zone of inhibited precipitation. Data of experiment B.

C. *The pH of minimum stability and the turbidity as a function of the concentrations of the interacting components*

This subject is illustrated by an experiment in which β-globulin and γ-globulin were mixed in different proportions. The total protein content was always the same. Each mixture was analysed with turbidimetry; the pH of minimum stability and the turbidity at this pH is shown in

TABLE 16. *Interaction between γ-globulin and β-globulin (experiment C).*

Fractions	Protein concentration	Ratio β/γ	pH of maximum turbidity	Turbidity
$\beta+\gamma$	1.8	0	7.4	12
		0.26	7.1	104
		0.61	6.9	155
		1.53	6.6	193
		3.43	5.5	216
		∞	5.3	211
$\beta+\gamma$	1.8	0	6.8	48
		0.26	6.7	67
		0.61	6.6	93
		1.53	6.1	124
		3.43	5.9	151
		∞	5.2	143

Table 16. When one of the components dominates, the pH of minimum stability of the mixture shifts nearer to the pH of this component. The normal β/γ ratio (0.7 or less) corresponds to a pH of minimum stability from about 6.7–7.0.

Comment.—This experiment once again demonstrates that the turbidity in the inter-isoelectric zone is often unexpectedly high and cannot be predicted by the turbidities shown by the isolated components at the same pH (see Fig. 13, p. 52, and Fig. 21). It also shows the shifting of the pH of minimum stability, already demonstrated in the "water dilution test with varying pH" (Fig. 5, p. 32), and, in addition, emphasises the importance of this pH in the discussion on the lability reactions (Fig. 6, p. 33). The shifting of the hydrogen concentration at maximum precipitation with varying proportions of the interacting proteins was already pointed out by Michaelis and Davidsohn in 1912. The importance of the interaction between β_1-lipoprotein and γ-globulin for the formation of euglobulin (the precipitate obtained at low ionic strength) has especially been emphasised by Gurd (1949). Normal serum, however, gives maximal precipitation below pH 6.0. According to the present author the interaction between γ-globulin and the α_1- and α_2-globulins must not be neglected (experiments D (1) and E). The effect of albumin is further discussed under E and F.

D. *Different parts of γ-globulin interacting with the same protein*

1. In some experiments electrophoretically prepared α_2-globulin was mixed with different parts of γ-globulin, the fast moving component γ_1, or the slowly moving γ_2, each in nearly the same protein concentra-

TABLE 17. *Interaction between γ-globulin and other serum proteins.*

Exp.	Fractions (all prepared by electrophoresis)	Protein concentration mg/100 ml	pH of maximum turbidity	Turbidity	Precipitate (μg protein/5 ml determined as γ-globulin)
D (1)	γ_1	2.0	6.3	93	47
	γ_2	1.7	7.5	22	28
	α_2	1.2	4.8	52	23
	$\alpha_2+\gamma_1$	1.2 + 2.0	5.3	211	71
	$\alpha_2+\gamma_2$	1.2 + 1.7	5.7	288	84
D (2)	γ_1	1.5	6.3	70	
	γ_2	1.2	7.3	32	
	alb. $+\gamma_1$	5.8 + 1.5	5.8	58	
	alb. $+\gamma_2$	5.8 + 1.2	5.8	43	
E	γ_1	1.9	6.5	111	53
	alb. $+\gamma_1$	7.1 + 1.9	6.3	100	35
	α_2	2.2	5.0	96	36
	$\alpha_2+\gamma_1$	1.9 + 2.2	5.6	314	81

tion. As is shown in Table 17, D (1), the interaction between α_2- and γ_2-globulin, resulting in a turbidity increase, is far more pronounced than between α_2- and γ_1-globulin. This effect is also shown by the precipitates. The experiment may be compared with the experiment with the mucoprotein (Table 18, F (2)) where a high turbidity was also obtained in a mixture with γ-globulin.

2. A somewhat different picture is seen when albumin is mixed with different parts of γ-globulin, as shown in Table 17, D (2). All protein fractions were prepared by electrophoresis. In the interaction between albumin and γ_1-globulin an unexpected effect is revealed, a *diminished* turbidity (as compared with the isolated γ-globulin), also observed in the inter-isoelectric zone. The difference in the interaction of albumin with γ_1- and γ_2-globulin is, however, shown clearly in addition by this experiment.

Comment.—These experiments demonstrate the complexity of the problem of these interactions. Of course, the γ-globulins, being heterogeneous with regard to solubility, molecular weights and isoelectric points will exhibit different interaction with other proteins. It seems to be an attractive explanation for the phenomenon described to assume that the greater the difference between the isoelectric points or the higher the charge of the colloids, the more intensive will be the interaction between the proteins. Applied to the lability reactions this finding would give a special importance to the observed increases of γ_2-globulin in some cases of hepatitis and cirrhosis (p. 65).

E. *Serum proteins with different isoelectric points interacting with the same γ-globulin*

Experiment D (2) initiated another experiment wherein the same γ-globulin was mixed with albumin or with α_2-globulin. The results are shown in Table 17, E. The usual mutual precipitation between the proteins is shown in the interaction between α_2-globulin and γ-globulin, at least if determined by turbidity. In the experiment between albumin and γ-globulin another effect is shown. As a result of the interaction the pH of minimum stability of the mixture is another than that obtained with isolated γ-globulin, but the turbidity is less than expected.

Comment.—The interaction between α_2-globulin and γ-globulin or in other words the participation of those proteins in the formation of euglobulin has particularly been studied by Svensson (1940). Already in 1905, however, Hardy observed the presence of carbohydrate in the precipitate obtained by dialysis of serum at acid reaction. Hewitt (1938) showed that a carbohydrate rich serum protein fraction, called globoglycoid, gave a heavy precipitate with a pseudoglobulin fraction. The globoglycoid was later identified as consisting mainly of α-globulin, and the pseudoglobulin as a γ-globulin, by Svensson (1940). There is no doubt that in serum, α_2-globulin in addition to β-globulin plays an important role in the interaction with γ-globulin, with its double aspect of mutual precipitation at one pH and inhibited precipitation at another. The presence of α_2-globulin in euglobulin has also been confirmed by Simon (1954) and Vargues (1955).

The experiments with albumin (D (2) and E) show a confusing result, somewhat opposed to those obtained before. The interaction between albumin and γ-globulin in these cases may be interpreted by assuming the existence of "complexes" between the two proteins, more soluble than γ-globulin alone, and occurring also in the interisoelectric zone. The effect of albumin in these cases can in a way be compared to the effect of neutral salts on solubility and pH of minimum stability of γ-globulin (pp. 58 and 60). The existence of such complexes has also been detected in electrophoresis experiments by Longsworth, Keith *et al.* in 1940 on mixtures of ovalbumin and ovomucoid and by Rice, Stahmann and Alberty in 1954, on mixtures of lysine polypeptides and bovine serum albumin. Tiselius in 1930 and Klezckovski in 1946 also point out the possibility of an interaction in the interisoelectric region even though no precipitate at all is obtained. After all, this is a question of solubility, "the charge cannot be the only factor of solubility, since many proteins are soluble at their isoelectric point "(Tiselius).

F. *Comparison between different albumins in mixture with γ-globulin*

In these experiments different albumins, ethanol-fractionated or prepared by electrophoresis, were compared in their interaction with ethanol-

TABLE 18. *Interaction between γ-globulin and albumin and between γ-globulin and mucoprotein.*

Z = prepared by zone electrophoresis; C = prepared by precipitation with ethanol at low temperature.

Exp.	Fractions	Protein concentration mg/100 ml	pH of maximum turbidity	Turbidity
F (1)	γC	1.0		very faint
	alb. C + γC	5.0 + 1.0	6.2	13
	alb. Z + γC	5.0 + 1.0	6.5	70
F (2)	mucoprotein (MP)	1.5	none	none
	γC	1.1		very faint
	MP + γC	1.5 + 1.1	5.2	102
	alb. C + γC	1.5 + 1.1		none

fractionated γ-globulin. In experiment F (1) (Table 18) the commercial dissolved preparations of albumin and γ-globulin were used after extensive dialysis against the veronal buffer. Here the electrophoretic albumin gave a very distinct turbidity in mixture with γ-globulin as compared with the slight precipitating effect of the ethanol-fractionated albumin. In F (2) (Table 18) a lyophilised preparation of albumin was used and compared in its effect with the mucoprotein. The difference between the two proteins in their interaction with γ-globulin is evident and ought to be compared with the difference in electrophoretic mobility between the two proteins. In another experiment (not tabulated) the presence of the (ethanol-fractionated and lyophilised) albumin could only be detected by a slight displacement of the precipitation curve of γ-globulin towards the acid direction (pH of maximum precipitation displaced from 6.9 to 6.4 with an albumin/γ-globulin ratio 2:1). Another lyophilised preparation of albumin, however, gave a precipitation curve with γ-globulin similar to the one presented in experiment B. Here again, the albumin–γ-globulin mixture had been stored for a few days before investigation.

Comment.—Earlier investigators (cf. Svensson 1940) have always claimed that albumin does not participate in the formation of euglobulin. On the other hand, in Cohn's system of fractionation of serum proteins (which, however, contains ethanol) albumin was precipitated together with γ-globulin at a pH more acid than 5.8 (Cohn 1953). Lontie and Morrison (1947) demonstrated a positive interaction between albumin and γ-globulin by means of light-scattering investigations; this, however, does not necessarily indicate the subsequent formation of a precipitate.

The experiments presented above and in experiments B, D (2) and E (Tables 15 and 17) give an example of the variability in the interaction

with γ-globulin obtained with different human albumins. The cause of this inconsistency is at present quite obscure, but may be found in such variables as different admixtures of α_1-globulin or mucoprotein or other qualitative differences in the albumins, for instance partial denaturation. The question of the effect of different concentrations of albumin on the interaction also needs to be more thoroughly investigated, as perhaps also the tendency to interaction effected by albumins carrying different amounts of bilirubin or other substances.

General discussion and conclusions

The experiments in this paper have been carried out with only a restricted number of lability reactions. But the conclusions to which they have given rise are, nevertheless, also applicable to some extent to other lability tests.

The general principles on which the results of the lability reactions depend are, of course, based on the common parameters of the solubility of serum proteins, pH, ionic strength and protein concentration. These parameters have been analysed on isolated protein fractions, and special attention has been directed to the pH of minimum stability (maximum turbidity) since this figure gives valuable information concerning the interacting proteins and their relative proportions. The particular effect of the increase of protein concentration on the yield of precipitate, and the effect of decreasing ionic strength on the displacement of pH of minimum stability, is re-emphasised. In many lability reactions, working with dilution series of serum in poorly buffered systems, these effects are often obscured and it is a Herculean task to bring order into the different mechanisms.

The dependence of the lability reactions on the electrophoretic serum protein fractions has been particularly emphasised in the earlier discussions with regard to albumin and γ-globulin, and even more fully confirmed by the experiments here described. But the fact that this dependence cannot possibly be said to furnish a complete and definitive explanation of the results of these reactions must be pointed out. The conditions obtained in the obstructive jaundice and acute hepatitis groups exemplify in a striking manner the validity of this conclusion, as do also the experiments with addition of isolated protein fractions to sera. Statistical considerations on the relationship between albumin and certain lability reactions also point in the same direction. It will, therefore, probably be necessary in future discussions to pay more attention to the globulins with an intermediary electrophoretic mobility and the modifying effect exercised by them on the precipitation of γ-globulin in the lability reactions than has hitherto been customary. It should be noted that the

lipoproteins and the glycoproteins, the effect of which on the lability reactions has been discussed in the literature in various contexts, belong to this category of globulin fractions.

Additional illustrative material regarding the manner in which this modifying effect is exerted is provided by the studies on the protein-protein interaction, performed with isolated serum protein fractions. The turbidity or the precipitate is to a considerable extent dependent on the pH. On the alkaline side of the pH of minimum stability of γ-globulin, albumin and the other globulins inhibit the precipitation of γ-globulin. In the inter-isoelectric zone, on the contrary, the same proteins exert an accelerating effect on the precipitation of γ-globulin, probably by co-precipitation. Some preparations of albumin, however, provide an exception as they exert a slightly inhibiting effect on the γ-globulin precipitation, even in the inter-isoelectric zone.

Particular stress is laid on the role played by the α-globulins in their interaction and co-precipitation with γ-globulin, as they participate in the formation of euglobulin, and also contain the bulk of glycoproteins. The interaction between β-globulin and γ-globulin has a special trend. The turbidity observed at the pH of minimum stability in a mixture of these two proteins, is to a great extent dependent on the high lipid content of the precipitate. As a matter of fact, these two effects, the inhibition of precipitation due to protein-protein interaction on one hand, and the turbidity increase due to high lipids in the precipitate on the other, may counteract each other, and consequently, the interpretation may be difficult if the precipitation is studied at a single, alkaline pH. This applies especially to a β-globulin with a high lipid content as often occurs in liver diseases. The question of the interaction between α_1-lipoprotein and γ-globulin needs further investigation, since the evidence given in the literature is contradictory (Bauer 1949, Cohn 1953).

The difference in interaction displayed by the slow- and fast-moving parts of γ-globulin is certainly of some significance. It must, however, be admitted that this effect is to some extent also counteracted by the difference in solubilities between these two components.

The precipitation curve of whole serum at low ionic strength and varying pH may thus be looked upon as mainly a result of interaction between albumin, α_1-globulin, α_2-globulin and β-globulin on one side and γ-globulin on the other. The shifting of pH of maximum turbidity with varying proportions of the interacting proteins gives a satisfying explanation of the different types of precipitation curves, shown by the modified water dilution test in various diseases. An increase of α_1- and α_2-globulins, as occurs in obstructive jaundice, will cause the pH of minimum stability (of the interacting proteins) to make a considerable shift in the acid direction. An isolated decrease of γ-globulin is followed by the same displacement, whereas an increase of γ-globulin will draw the pH of minimum stability nearer to the isoelectric region of this protein. The

same effect may, however, also be exerted by a decrease of albumin, α_1-, α_2- and β-globulin, such as occurs in cases of advanced liver parenchymal injury.

The statistical studies on the correlation between the electrophoretic serum protein fractions and some lability reactions are in accordance with these results. The modifying influence exerted by other globulins on the precipitation of γ-globulin is more pronounced in some tests and less so in others, and can even show completely divergent results from test to test. One of the most important factors which regulate the extent of this divergence is the pH at which the lability reactions are carried out, a fact that stands out clearly in the light of the studies on protein-protein interaction. The same protein, depending on the pH, can thus inhibit *or* enhance the precipitation of γ-globulin.

The discussion has hitherto been restricted to the protein-protein interaction, but in the survey of the literature several examples were given of protein interaction with non-protein charged macromolecular substances. The effect exerted by lecithin (and other phosphatides?) on the solubility of proteins (Feinschmidt 1912, Bungenberg de Jong 1931), for instance γ-globulin (Adner, unpublished investigations), seems to be of the same nature as the globulin-globulin interaction described above, and may perhaps be of some importance in connection with the lability reactions (cf. Popper, de la Huerga *et al.* 1950).

The mechanism of a test like the water dilution test may, consequently, be interpreted as follows: The diminution of ionic strength brings about (1) an increased readiness of the lipoproteins to precipitate; (2) an increased tendency to interaction between γ-globulin and other serum proteins resulting in an increased yield of precipitate (at least at a pH below 7); (3) a shifting of the pH of minimum stability of all globulins, but first and foremost of γ-globulin in an alkaline direction. Other precipitating agents, used in the lability tests, as for instance certain metal ions, lipid emulsions, etc., have partly been studied already in their general effect on proteins, although the knowledge has not as yet been applied to the lability reactions. In most cases, however, it is the question of specific interaction with certain serum proteins, of a kind shortly referred to on p. 59.

The fact that sera from cases of liver parenchyma disease so often give positive reactions is, therefore, dependent on a combination of numerous factors, of which the following will be specially emphasised.

1. An increase of γ-globulin. This cannot, however, be the significant factor which differentiates the acute hepatitis from the obstructive jaundice groups, in which lability tests are mainly used as aids to differential diagnosis.

2. A decrease of albumin. This protein cannot, however, be regarded as a factor of such vital importance in this connection as it was previously considered to be, for the reasons cited above.

3. A normal concentration or a decrease in α_1-, α_2- (especially the more rapidly moving section of α_2-globulin?) and occasionally β-globulin. The probability that these fractions have an inhibitory effect on the precipitation of the γ-globulin in certain lability reactions has been supported by statistical investigations and corroborated by experiments with isolated fractions.

4. These changes, *in conjunction with an increased lipid content in the β-globulin*, and also, perhaps, qualitative alterations in the lipids comprising this fraction should have a very significant effect.

Earlier research workers have postulated the presence, in liver parenchymal diseases, of albumins and γ-globulins which show divergencies from the normal in their behaviour in the lability reactions. This delicate question has only been slightly touched upon in this investigation. There are, in addition, certain disparities between the lability reactions and the electrophoretic picture within the group comprising parenchymal diseases (acute hepatitis and cirrhosis of the liver) to which this explanation can scarcely be applied. The observation of a γ-globulin with a predominance of the slowly-moving component in some cases of liver parenchyma diseases is, however, in accordance with these earlier reports. An increase in this fraction involves a considerable extension of the interaction with more rapidly moving serum proteins, and thus, in addition, an increase of the precipitate. With regard to albumin, it has already been pointed out that even normal albumins from different sources display a great variability in their tendency to interaction with γ-globulin. These problems have to be attacked with improved methods for isolating the protein fractions, combining high resolving power with reproducibility.

SUMMARY

Part I

The survey of the literature in Chapters I and II A is summarised on pages 14 and 19.

Chapter II

C.—Paper electrophoretic data from several groups of liver diseases are worked through and correlated with the flocculation reactions (thymol, cephalin–cholesterol flocculation and Takata reactions) simultaneously performed. A comparison between the electrophoretic patterns obtained from infective hepatitis and obstructive jaundice shows clearly that the main difference between these two groups is to be found in the concentrations of α_1- and α_2-globulins, which are elevated in obstructive jaundice. The concentration of γ-globulin is increased in both groups, though somewhat more distinctly in the hepatitis series. Crucial importance cannot, however, be attached to this difference, since both groups here diverge from normality in the same direction. The concentration of albumin is subnormal, and its incidence is the same in both groups. It must therefore be concluded that those fractions (albumin and γ-globulin) which have been first and foremost discussed in connection with the flocculation reactions, cannot alone have caused the positive results in the hepatitis group. Attention should be more directly focused on the globulins with intermediary electrophoretic mobility. A comparison between the hepatitis and cirrhosis groups demonstrates that the results of some flocculation reactions are as a rule higher in the hepatitis group if the figures are grouped after the degree of γ-globulin increase. The electrophoretic data presented give no explanation of this phenomenon. A possible cause may, however, be found in qualitative and/or quantitative differences in the lipoproteins. A tendency to a decreased content of α_2-globulin (hepatitis) and of α_1-globulin and β-globulin (cirrhosis) is discernible in the groups of liver parenchyma disease.

D.—By statistical studies the considerable dependence of the results of the flocculation reactions (the thymol, zinc sulphate and water dilution tests) on the concentration of γ-globulin is confirmed. The correlation between the tests and γ-globulin is, however, not strictly linear, at least as regards the thymol test. The correlation with albumin is on the same low level as that with the globulins with an intermediary electrophoretic mobility. In some flocculation reactions the effect of these

globulins on the protein precipitation seems to be promotory, in other reactions, on the contrary, it appears to have an inhibitory tendency, and this difference is probably closely connected with the pH of the test.

E.—Lyophilised samples of albumin and γ-globulin (ethanol-fractionated) have been added to two sera; one, with a low albumin content, from a case of liver cirrhosis, and the other from a case of agammaglobulinemia. The strong promoting effect of γ-globulin on the precipitation is shown, although the response in the thymol test is not linear to the increase of γ-globulin content. The inhibiting effect of albumin is also evident, although perhaps not so strong as might have been expected. In the thymol reaction the albumin inhibition is pronounced only at high figures of the albumin/γ-globulin ratio. The inhibiting effect of the globulins with intermediary electrophoretic mobility is, however, indirectly demonstrated when the results of the two experiments are combined.

Part II

This part deals with a direct study of the effect of the interactions between isolated serum proteins on their solubility, and gives some physico-chemical aspects on the flocculation reactions.

Chapter I

The investigated proteins have been isolated by preparatory electrophoresis on paper or starch as stabilising medium. As a model experiment the author has modified the water dilution test on a microscale, and in connection with the method the conception of turbidity is discussed. It is especially emphasised that a turbidimetric study of precipitation only gives a true picture of the course of aggregation initially. This matter is illustrated in some experiments, where the increase of turbidity is followed for some period of time. With the technique devised (protein precipitation at low ionic strength and varying pH) symmetrical precipitation curves are obtained for the protein fractions with a characteristic pH of maximum turbidity.

Chapter II

The fundamental parameters of protein solubility, namely pH, ionic strength and protein concentration are discussed.

Protein concentration.—It is shown in the precipitation experiments that there is no strictly linear correlation between the turbidity obtained and the original protein concentration, and this applies also to the thymol and zinc sulphate reactions. With increasing protein concentration the yield of precipitate, at least if evaluated as turbidity, is increased proportionately to a still greater degree. These observations are relevant to the solubility criteria on pure and homogeneous proteins.

Ionic strength.—A decrease in ionic strength generally gives an increasing precipitation of the globulins investigated. But the α_2- and β-globulins are more easily precipitable (less stable) than γ-globulin if studied at their pH of minimum stability.

pH.—When dealing with lability reactions performed at low ionic strength, particular importance is laid on the shift of pH of minimum stability into the alkaline direction which occurs when the ionic strength is diminished. This tendency is especially pronounced in γ-globulin. The pH of minimum stability at ionic strength 0.001 is given for the main globulin fraction. Observations are reported indicating that in some cases of liver parenchyma damage the main globulin increase concerns its slowly moving component (γ_2). This finding explains the earlier observations of a labile γ-globulin in liver injuries.

Chapter III

In order to calibrate the turbidimetry, the precipitate is spun down and computed as microgram protein. The ratio turbidity/μg protein varies in the precipitate obtained from the different globulin fractions, β-globulin giving the highest values. These variations are principally ascribed to the content of the lipoproteins in the precipitate, since the lipids make a considerable contribution to the turbidity. The lack of correlation between protein electrophoresis and flocculation reactions is very well accounted for by this fact.

The percentage of precipitated protein at low ionic strength is higher in the faster moving component of γ-globulin than in the slow one, probably because of the presence of high-molecular globulins.

Chapter IV

The modifying effect exerted by the interaction of other proteins or macromolecular substances present on the solubility of single proteins has been thoroughly studied by protein chemists for more than 50 years. A survey of literature is given, and the author applies this principle to flocculation reactions in model experiments where isolated serum proteins are mixed with each other and turbidimetry at low ionic strength is performed. There is definite documentary evidence for the presumption that the serum proteins possessing an isoelectric point within the pH range of 4.5 to about 6 (albumin, α_1-globulin, α_2-globulin and β-globulin) may strongly interfere with the solubility of γ-globulin. This interference is principally of two kinds; depending on the pH at which the precipitations is performed. With the exception of some preparations of albumin the presence of those proteins mentioned decreases the solubility of γ-globulin (increases the precipitation) at a pH lying in the interval between the isoelectric points of the interacting proteins. On the alkaline side of the isoelectric point of γ-globulin, the solubility of this protein is instead increased by the other interacting

proteins. According to the pH, the same protein may thus exert an inhibiting or accelerating effect on the precipitation of γ-globulin. The participation, not only of β-globulin but also of α_2-globulin and the mucoproteins in the formation of "euglobulin" is demonstrated. Different parts of the γ-globulin show variations in the strength of the tendency to interact with the other proteins.

As demonstrated in experiments with mixtures of β-globulin and γ-globulin, the interaction between lipoproteins and γ-globulin is sometimes difficult to interpret, especially if the precipitate is evaluated with turbidimetry. The lipid component of the lipoprotein may increase, and the protein component inhibit, the precipitation. The presence of phosphatides may produce still more complicated results.

The inhibition of the precipitation is explained by assuming the formation of soluble "complexes" between the interacting proteins. With some preparations of albumin, however, results have been obtained which indicate that such complexes also occur in the inter-isoelectric interval.

The turbidity curve of whole serum obtained at low ionic strength and varying pH is evidently a result of this protein-protein interaction. When dealing with flocculation reactions performed at a single pH the conception of pH of maximal turbidity and its variations with the relative proportions of the interacting proteins must be kept in mind, otherwise the results are difficult to interpret.

In a final section an attempt has been made to combine the results in part I and II with special regard to the high percentage of positive results in the flocculation reactions in liver parenchyma diseases.

APPENDIX

Table I

The cases belonging to group 2, acute infective hepatitis (p. 24), correspond to the following numbers in hospital records. All cases were from the Hospital of Infectious Diseases, Uppsala, except two, who were under the supervision of a general practitioner.

707/55	48/56	58/56	74/56
23/56	49	65	78
31	50	66	79
32	51	68	245/56
36	52	69	
46	55	70	
47/56	57/56	73/56	

Table II

The cases belonging to group 3, obstructive jaundice (p. 24), correspond to the following numbers and diagnoses in hospital records. All cases were from the Surgical Ward, University Hospital, Uppsala.

Diagnosis	No.	Additional diagnosis
Acute cholecystitis	1878/56	cholelithiasis
	1902	acute pancreatitis, pneumonia
	2146	acute pancreatitis
	2366	
	2450	
	2573/56	
Chronic cholecystitis	1892/56	
	1928	
	1961	
	1988	
	2000	
	2039	bronchopneumonia
	2306	
	2422/56	choledocholithiasis

Table II (cont.)

Diagnosis	No.	Additional diagnosis
Cholelithiasis	1836/56	
	1874	
	1911	
	1954	acute bronchitis
	2085	
	2412	acute pancreatitis
	2431	choledocholithiasis
	2555/56	
Other diseases of the bile ducts	2360/56	obstructive? jaundice (unspecified)
	2598	gall bladder disease
	2599/56	stricture of the ampulla of Vater; chronic pyelonephritis
Malignant disease	1905/56	carcinoma of the peritoneum
	1979	carcinoma of the ampulla of Vater
	2468/56	gastric carcinoma in advanced stage

Table III

The cases belonging to group 4 (liver cirrhosis) (p. 24) correspond to the following numbers and diagnoses in hospital records.

Case no.	Additional diagnosis	Case no.	Additional diagnosis
M 490/56	dipsomania	P 449/56	
M 707/56	chronic hepatitis	P 468/56	
M 1703/56		P 598/56	insufficiency of pancreas
M 1678/56	chronic alcoholism		
M 1359/56		P 393/56	
M 1577/56		P 115/57	
M 546/56		P 123/57	
M 1584/56	delirium tremens	P 521/57	subacute hepatitis, hypersplenism
M 1137/56	diabetes mellitus		
M 1862/55		P 552/56	
M 2232/56			
M 340/57			
M 1210/57	chronic hepatitis		
MP 2844/57	chronic hepatitis		
E 324/57			

M Medical Ward.
P Childrens' Ward.
MP Outpatients' Department of Medicine, University Hospital, Uppsala.
E Hospital of Infectious Diseases, Uppsala.

Table IV

Diagnoses of cases, whose sera were used as material in part I, chapter II D.

Diagnosis	No. of cases	Diagnosis	No. of cases
Liver cirrhosis	4	Tuberculosis	3
Infectious hepatitis	4	Renal disease	3
Obstructive jaundice	3	Duodenal ulcer	1
Bronchopneumonia	3	Cushing's disease	1
Ulcerative colitis	1	Pulmonary embolism	1
Malignant disease	8	Heart failure	1
Polycythemia	1	Hypertonia	3
Aplastic anemia	1	Myocardial infarction	7
Rheumatic fever	1	Diabetes	8
Rheumatoid arthritis	3	Miscellaneous	5

Table V

The case numbers correspond to the following numbers in hospital records.

Case no.	Record no.	Diagnosis	Thymol turbidity
1	CS 242/57	acute cholecystitis	0.01
2	E 377/56	serum hepatitis	0.36
3	M 2232/56	liver cirrhosis	0.88
4	P 393/56	liver cirrhosis	0.56
5	M 670/56	hypogammaglobulinemia	0
6	F 53	liver cirrhosis	0.79
7	M 1061/53	acute hepatitis	0.49
8	M 2/55	subacute hepatitis	0.64
9	S 362/54	acute hepatitis	1.16
10	E 346/54	chronic hepatitis	0.77
11	K 213/57	chronic cholecystitis	0.01
12	E 115/56	acute hepatitis	0.99
13	M 558/56	myeloma	1.20
14	M 2553/53	acute hepatitis	0.60
15	M 2499/53	myocardial infarction	0.16
16	M 2528/53	nephrolithiasis, pyelonephritis	—

M Medical Ward.
K Surgical Ward.
CS Chest Clinic.
P Childrens' Ward, University Hospital, Uppsala.
E Hospital of Infectious Diseases, Uppsala.
S Medical Department, Hospital of Sala.
F Medical Department, Hospital of Falun.

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